

FINAL REPORT

Bioremediation Approaches for Treating Low Concentrations of *N*-Nitrosodimethylamine in Groundwater

SERDP Project ER-1456

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14. ABSTRACT NDMA is a potent carcinogen and an emerging groundwater pollutant. The objective of this SERDP project was to study NDMA biodegradation and to develop in situ and ex situ bioremediation strategies for the compound. A detailed summary of the relevant findings for this project is provided in the Executive Summary of this report.					
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Acronyms and Abbreviations

ASTDR—Agency for Toxic Substances and Disease Registry
ATU—allyl thiourea
BSM—basal salts medium
CDHS—California Department of Health Services
 CH_2Cl_2 —dichloromethane (methylene chloride)
 CH_3OH —methanol
 CO_2 —carbon dioxide
DCM—dichloromethane (methylene chloride)
DHS ELAP—Department of Health Services Environmental Laboratory Accreditation Program
DMF—N, N-dimethylformamide
DNA—deoxyribonucleic acid
DO—dissolved oxygen
DoD—Department of Defense
EI—e
EPA—Environmental Protection Agency
ES—e Figure 11
FBR—fluidized bed bioreactor
Fe—iron
FY—fiscal year
g—grams
GAC—granular activated carbon
GC—gas chromatograph
GC/MS—gas chromatography/mass spectrometry
HCl—hydrochloric acid
HCHO—formaldehyde
HCOO—formate
HPLC—high performance liquid chromatography
hr—hours
HRMS/MS—high resolution mass spectrometry/mass spectrometry
HRT—hydraulic residence time
 H_2SO_4 —sulfuric acid
KI—potassium iodide
 KNO_2 —potassium nitrite
L—liters
MBR—membrane bioreactor
 μCi —microcurie
mCi—millicurie
MDL—method detection limit
MeOH—methanol
 μg —micrograms

Acronyms and Abbreviations (*continued*)

mg—milligrams
mL—milliliters
 μ M—micromolar
mM—millimolar
MMO—methane monooxygenase
mmol—millimolar
Mn—manganese
MNO₂—manganese dioxide
MTBE—methyl *tert*-butyl ether
N—normal
NADP—nicotinamide adenine dinucleotide phosphate
NaOH—sodium hydroxide
NaSO₄—sodium sulfate
NDAB—nitro-2,4-diazabutanal
NDMA—N-nitrosodimethylamine
(NH₄)₂HPO₄—diammonium phosphate
(NH₄)H₂PO₄—ammonium phosphate
nM—nanomoles
NMP—1-methyl-2-pyrrolidinone
NO—nitrous oxide
NO₂—nitrite
NO₃—nitrate
NTDMA—N-nitrodimethylamine
NTMA—N-nitromethylamine (methylnitramine)
O₂—oxygen
OD—optical density
OEHHA—Office of Environmental Health Hazard Assessment
pA—picoAmperes
PCR—polymerase chain reaction
PMO—propane monooxygenase
ppb—parts-per-billion
ppt—parts-per-trillion
PQL—practical quantitation limit
QPR—quarterly progress report
QRT-PCR—quantitative reverse transcriptase polymerase chain reaction
RL—reporting limit
RPM—rounds per minute
RNA—ribonucleic acid
RT-PCR—reverse transcriptase polymerase chain reaction
SDMO—soluble diiron monooxygenase
SERDP—Strategic Environmental Research and Development Program

Acronyms and Abbreviations (*continued*)

SRT—solids retention time

TCE—trichloroethene

TDO—toluene dioxygenase

T₂MO—toluene-2-monooxygenase

T₄MO—toluene-4-monooxygenase

TSB—tryptic soy broth

UDMH—1,1- dimethylhydrazine

USEPA—United States Environmental Protection Agency

UV—ultraviolet

v/v—volume/volume

VOA—volatile organic analysis

VOC—volatile organic compound

WSTF—White Sands Test Facility

WWTP—wastewater treatment plant

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Executive Summary

N-Nitrosodimethylamine (NDMA) is a potent carcinogen and an emerging groundwater and drinking water contaminant in the United States. NDMA contamination of groundwater at military and aerospace sites stems largely from the former use and disposal of liquid rocket propellants containing 1,1-dimethylhydrazine (1,1-DMH or UDMH). This compound, which is a major component of the propellant Aerozine-50, contains NDMA as a chemical impurity and has also been observed to oxidize to NDMA in natural environments. Military and aerospace facilities reporting NDMA in groundwater include the Rocky Mountain Arsenal (CO); Air Force Site PJKS (CO); White Sands Missile Range (NM); Aerojet Corp (multiple locations in CA); Jet Propulsion Labs (CA) and Edwards Air Force Base (CA). NDMA is also formed as a disinfection byproduct in drinking water and wastewater treated with chloramines.

There is presently no federal maximum contaminant level (MCL) for NDMA in drinking water. However, the chemical is a potent animal carcinogen and a suspected human carcinogen, so its presence in drinking water represents a public health concern. In December 2006, the California Office of Environmental Health Hazard Assessment (OEHHA) established a public health goal (PHG) for NDMA in drinking water of 3 parts-per-trillion (ppt or ng/L) based on risk calculations. The development of a PHG is a key step in the establishment of an MCL for an unregulated contaminant in California. In addition, the U.S. Environmental Protection Agency (USEPA) recently added NDMA and four other nitrosamines to its Unregulated Contaminant Monitoring Rule 2 (UCMR 2). As a result, many large water utilities are now required to monitor for NDMA.

The most widely used treatment technology for removing NDMA from groundwater to ng/L concentrations is ultraviolet irradiation (UV). This approach is effective, but it is also expensive, requiring pump-and-treat infrastructure and a medium or low-pressure UV system. In addition to capital costs, the energy input to reduce NDMA concentrations by one order of magnitude is approximately ten times that necessary for standard disinfection of viruses and other water-borne pathogens. At some military sites, reductions in NDMA concentrations of 4 log orders (e.g., from 30 µg/L to 3 ng/L) may be required to meet treatment objectives. Less expensive *in situ* or *ex situ* treatment alternatives are not presently available.

The objective of this SERDP project was to study NDMA biodegradation and to explore potential *in situ* and *ex situ* bioremediation strategies for this contaminant. The following research areas were examined: (1) the pathways of NDMA oxidation by propane- and toluene-oxidizing strains were determined; (2) NDMA biodegradation was quantified in microcosms prepared from aquifer samples and in other natural environments under both aerobic and anaerobic conditions; (3) indigenous strains capable of aerobic, co-metabolic NDMA metabolism were isolated and identified from aquifer samples; (4) the technological viability of NDMA treatment using an advanced bioreactor design was assessed; (5) the possibility of treating both NDMA and chlorinated solvents in a single bioreactor was examined; and (6) the potential for *in situ* treatment of NDMA and commingled NDMA and TCE using biostimulation and bioaugmentation was

evaluated. The data provide fundamental information on NDMA biodegradation and a comprehensive evaluation of potential *in situ* and *ex situ* NDMA bioremediation options in the field.

Initial experiments revealed that propanotrophs and specific toluene-oxidizing strains were capable of metabolizing NDMA, and that these bacteria utilize different routes of metabolism. The toluene-oxidizer *Pseudomonas mendocina* KR1 was observed to oxidize NDMA primarily through a demethylation pathway, forming N-nitromethylamine (NTMA) and formate as terminal products. The enzyme toluene-4-monooxygenase (T4MO) was determined to be responsible for the initial oxidative step through studies with a T4MO clone. In contrast, the propanotroph *Rhodococcus ruber* ENV425 was found to transform NDMA primarily via a denitrosation route, producing nitric oxide, nitrite, nitrate, formate, methylamine and dimethylamine as degradation products. Unlike strain KR1, *R. ruber* ENV425 also produced a significant quantity of carbon dioxide during NDMA biotransformation. Strain ENV425 was observed to use NDMA for N in the absence of other available sources, but neither strain (ENV425 or KR1) was capable of utilizing NDMA as a sole source of carbon and energy. Rather, they each appear to transform NDMA co-metabolically during growth on propane (ENV425) or toluene (KR1) as primary substrates

For *in situ* or *ex situ* biological treatment of NDMA to be practical, $\mu\text{g/L}$ concentrations of the nitrosamine must be reduced to low ng/L levels. Very few compounds have such stringent treatment requirements, and biodegradation processes are rarely considered for such applications. However, batch experiments with strain ENV425 revealed that, when grown on propane, the bacterium could reduce NDMA from $8 \mu\text{g/L}$ to $< 2 \text{ ng/L}$ (the MDL for NDMA). Based on this initial finding, a laboratory reactor system was constructed to evaluate the potential for *ex situ* biological treatment of NDMA. The membrane bioreactor (MBR) was seeded with *R. ruber* ENV425 and received propane as the primary growth substrate. Oxygen was provided to the MBR in excess. At influent NDMA concentrations ranging from $\sim 8 - 80 \mu\text{g/L}$ in water, effluent concentrations of the nitrosamine were generally below 10 ng/L during more than 150 days of MBR operation. These laboratory data suggest that *ex situ* biological treatment of NDMA to ng/L concentrations is both feasible and sustainable. The addition of trichloroethene (TCE) to the reactor resulted in a significant increase in NDMA in the reactor effluent, most likely due to cell toxicity from TCE-epoxide. Thus, the joint treatment of NDMA and chlorinated solvents, such as TCE, in a single reactor is unlikely to be successful. However, technologies such as air-stripping can easily be implemented to remove chlorinated solvents from a groundwater stream prior to NDMA treatment in a bioreactor. Further studies are required to evaluate the cost and performance of a propane-fed bioreactor for NDMA treatment at the field scale.

In addition to pure culture and bioreactor studies, microcosm experiments were performed to evaluate the potential for *in situ* remediation of NDMA. Aquifer samples were collected from military (or former military) sites in CO, NJ, and CA for use in these studies. NDMA biodegradation was also evaluated in samples of surface soil, sludge, pond sediment, and manure. NDMA mineralization (conversion of ^{14}C -NDMA to $^{14}\text{CO}_2$)

was observed in surface soil, sludge, manure and other organic-rich environmental samples under aerobic conditions. NDMA degradation in these samples is hypothesized to be largely co-metabolic, with organisms growing on native organic compounds and fortuitously oxidizing NDMA. No bacterial strains capable of growing on NDMA as a sole carbon and energy source were isolated from any of the environmental samples.

In aquifer samples, pre-incubation with propane and oxygen for 2-3 weeks resulted in the rapid mineralization of ~ 50 µg/L of ¹⁴C-NDMA to ¹⁴CO₂. Significant NDMA mineralization was also observed in some samples receiving yeast extract, but not in those receiving oxygen only or in killed controls. Further studies with large-scale microcosms prepared from aquifer samples revealed that native propanotrophs were able to reduce NDMA concentrations from µg/L to ng/L concentrations after biostimulation with propane and oxygen. *Nocardioides* spp. were enriched on propane and isolated from two of the three aquifers. After growth on propane, each of these cultures (3 total) readily degraded NDMA. In addition, the impact of TCE on NDMA degradation was generally less pronounced in the aquifer microcosms than observed in the MBR study. In fact, both NDMA and TCE were biodegraded simultaneously in propane-amended samples from the NJ site. Even an initial concentration of 1 mg/L, TCE did not impact the rate or extent of NDMA biodegradation. The results suggest that *in situ* addition of propane and oxygen may be a viable remedial option for NDMA in groundwater at many sites with this nitrosamine.

For future remedial applications, the key findings of this project are as follows: (1) a variety of propane-oxidizing bacteria are capable of degrading NDMA to innocuous products, (2) these bacteria are widely distributed in groundwater aquifers and can be stimulated through the addition of propane and oxygen, and (3) biodegradation of the nitrosamine to low ng/L concentrations is feasible. The data provide potential options for both *in situ* and *ex situ* biological treatment of NDMA. For *ex situ* treatment, propane-fed bioreactors hold promise. The design, cost and long-term performance of this technology remains to be determined at field scale. For *in situ* treatment, application of propane and oxygen to groundwater is suggested as a possible remedial alternative. These gases can be applied through several methods including (1) air- and propane-biosparging, (2) groundwater recirculation with above-ground propane and oxygen addition, (3) bubble-free gas injection systems, and (4) trenches with air and propane injection lines. Field demonstration of one or more these techniques for NDMA treatment is required. However, if successful, this *in situ* approach is likely to be both cost effective and widely applicable at DoD sites.

1.0 PROJECT OBJECTIVES

The main objective of this SERDP project was to develop viable *in situ* and *ex situ* bioremediation strategies for treating groundwater contaminated with low concentrations of NDMA. The studies build upon previous research in our laboratory showing that specific strains of propane- and toluene-oxidizing bacteria can degrade NDMA, and that both *ex situ* reactors and *in situ* biostimulation may be successful approaches for treating this groundwater contaminant. During this SERDP Project, the following research areas were examined: (1) the pathways of NDMA oxidation by propane- and toluene-oxidizing strains were determined; (2) NDMA biodegradation was quantified in microcosms prepared from aquifer samples and in other natural environments under both aerobic and anaerobic conditions; (3) indigenous strains capable of aerobic, co-metabolic NDMA metabolism were isolated and identified from aquifer samples; (4) the technological viability of NDMA treatment using an advanced bioreactor design was assessed; (5) the possibility of treating both NDMA and chlorinated solvents in a single bioreactor was examined; and (6) the potential for *in situ* treatment of NDMA using biostimulation and bioaugmentation was determined. The data from this project provide basic information on NDMA biodegradation and a comprehensive evaluation of potential *in situ* and *ex situ* NDMA bioremediation options in the field.

2.0 BACKGROUND

The origin of NDMA in groundwater and drinking water includes industrial, agricultural, water treatment, and military sources. Contamination of military installations, NASA facilities, and aerospace contractors with NDMA has occurred largely from the use and disposal of 1,1-dimethylhydrazine (UDMH), a liquid rocket fuel that contained NDMA as an impurity and has also been found to oxidize to NDMA in the environment (Fleming et al., 1996; Mitch et al., 2003). Recent reports suggest that NDMA is widespread at military sites including the Rocky Mountain Arsenal (Fleming et al., 1996; Gunnison et al., 2000) and former Air Force Plant PJKS (PJKS; USEPA 2004, 2006). Aerospace contractors also have extensive plumes of NDMA in groundwater at sites in California (Girard, 2000). As a result, both Los Angeles and Orange Counties in California have found NDMA in groundwater supply wells during the past few years (CDHS, 2008a).

NDMA is a potent carcinogen and thus, its presence in groundwater, even at low concentrations, is of concern (National Toxicology Program, 2000). The State of California established an *action level* for NDMA in drinking water of 20 ng/L (i.e., part-per-trillion; ppt) in 1998, and then revised this level to 10 ng/L in March 2002 (CDHS, 2008b). Only three other compounds (of ~ 80 with regulatory action levels in California) are regulated at or below 10 ng/L (CDHS 2008a). The current drinking water regulation in Canada is 9 ng/L, and according to EPA, a safe level of NDMA in drinking water based on lifetime *de minimus* risk calculations ($< 10^{-6}$ risk of developing cancer) is only 0.7 ng/L. Recently, in December 2006, the California Office of Environmental Health Hazard Assessment (OEHHA) issued a public health goal for NDMA of 3 ng/L (OEHHA, 2006). Thus, regulatory levels for this compound in California may be reduced further in the future. Achieving clean-up levels for NDMA and other water-soluble organic pollutants to the low part-per-trillion range is a difficult technological challenge that has not received extensive study. Current technologies for treating NDMA in water include ultraviolet irradiation (UV) and adsorption to granular activated carbon (GAC). However, these methods are very expensive and/or ineffective for removing NDMA to required levels (Fleming et al., 1996; Gui et al., 2000). The objective of this SERDP project was to evaluate *in situ* and *ex situ* biological remediation options for the treatment of NDMA.

The effective treatment of NDMA in groundwater requires that the concentrations of the compound be reduced from a few to several hundred $\mu\text{g/L}$ to ng/L concentrations. In many instances, bacteria have been observed to have a lower threshold concentration for an organic substrate below which degradation ceases (Alexander, 1994). One theory for this threshold phenomenon is that as the concentration of a substrate decreases during degradation, a point is reached in which the energy required to maintain a bacterial cell is no longer met by the quantity of substrate available (Schmidt et al., 1985). At this point cells die and degradation ceases leaving a residual substrate (i.e., organic contaminant). Lower threshold values vary appreciably by compound and cell type; concentrations

ranging from approximately 0.0015 to 100 µg/L have been reported (Alexander, 1994). This threshold phenomenon is an important consideration when evaluating bioremediation strategies for NDMA. It is unlikely that a bacterial strain will be able to grow on a few µg/L of NDMA and reduce its concentration to ng/L levels. However, the degradation of NDMA by a co-metabolic process in which the bacterium actually grows on a secondary substrate (such as propane, toluene, butane, etc.) and degrades NDMA fortuitously, may allow threshold limitations to be overcome, and low concentrations to be achieved. Previous results utilizing a toluene-fed chemostat reactor suggest that this principle is correct (see next paragraph).

This SERDP project builds and expands upon previous research conducted in our laboratory examining the co-metabolic biodegradation of NDMA by propane- and toluene-oxidizing bacteria. The project, which was funded through a grant from the National Science Foundation (DMI-0212405) revealed that specific strains of propane- and toluene-oxidizing bacteria can co-metabolically degrade NDMA (Table 1). Experiments with a microbial strain containing cloned toluene-4-monoxygenase (T4MO) suggested that this enzyme is responsible for the initial oxidation of NDMA by the toluene-oxidizing strains (Figure 1). A chemostat reactor was set-up to assess the potential for *ex situ* biological treatment of NDMA. The chemostat was seeded with the toluene-oxidizer *P. mendocina* KR1, operated at a dilution rate of 0.02 hr⁻¹, and fed toluene (~ 2 g/day) as a primary growth substrate. NDMA was supplied to the reactor in the influent at concentrations ranging from 25 to 250 µg/L, to represent typical groundwater levels of the contaminant. Effluent levels from the NDMA reactor were generally below 0.2 µg/L during the 3½-month study (Figure 2). A similar reduction in NDMA levels was not observed in a control reactor that was not seeded with strain KR1. These data prove concept that NDMA can be consistently biodegraded to sub-µg/L concentrations during co-metabolic operation of a simple bioreactor. In addition to the reactor studies, a few experiments were performed previously to examine the potential for *in situ* treatment of NDMA in groundwater. The experiments conducted during this SERDP project build upon the results from the NSF effort. This project is designed to provide basic information on NDMA biodegradation and a comprehensive evaluation of potential *in situ* and *ex situ* NDMA bioremediation options in the field.

Table 1. Evaluation of NDMA biodegradation by different cultures.

Strain	Growth Substrate	Enzyme ²	NDMA Degradation
<i>Rhodococcus ruber</i> ENV425	propane	PMO	+
Strain ENV420	propane	PMO	-
<i>Methylosinus trichosporium</i> OB3b	methane	MMO	+
<i>Methylosinus sporium</i> ATCC 35069	methane	MMO	+
<i>Pseudomonas putida</i> F1	toluene	TDO	-
<i>Pseudomonas mendocina</i> KR1	toluene	T4MO	+
<i>Burkholderia cepacia</i> G4	toluene	T2MO	-
<i>Pseudomonas</i> sp. ENVPC5	toluene	T4MO	+
<i>Pseudomonas putida</i> ATCC 17453	camphor	P-cam 450	-
<i>Hydrogenophaga flava</i> ENV735	MTBE	NA ¹	-

¹ NA; data not available.

² PMO – propane monooxygenase; MMO – methane monooxygenase; TDO – toluene dioxygenase; T4MO – toluene-4-monooxygenase; T2MO – toluene-2-monooxygenase.

Figure 1. Biodegradation of NDMA by *P. putida* PPO200 (wild type) and *P. putida* AF (T4MO clone).

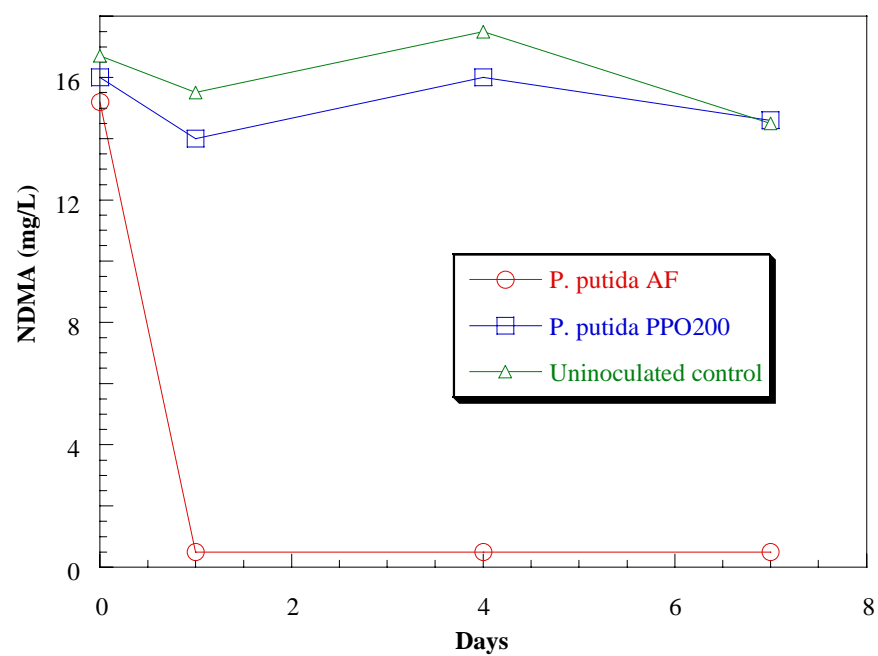
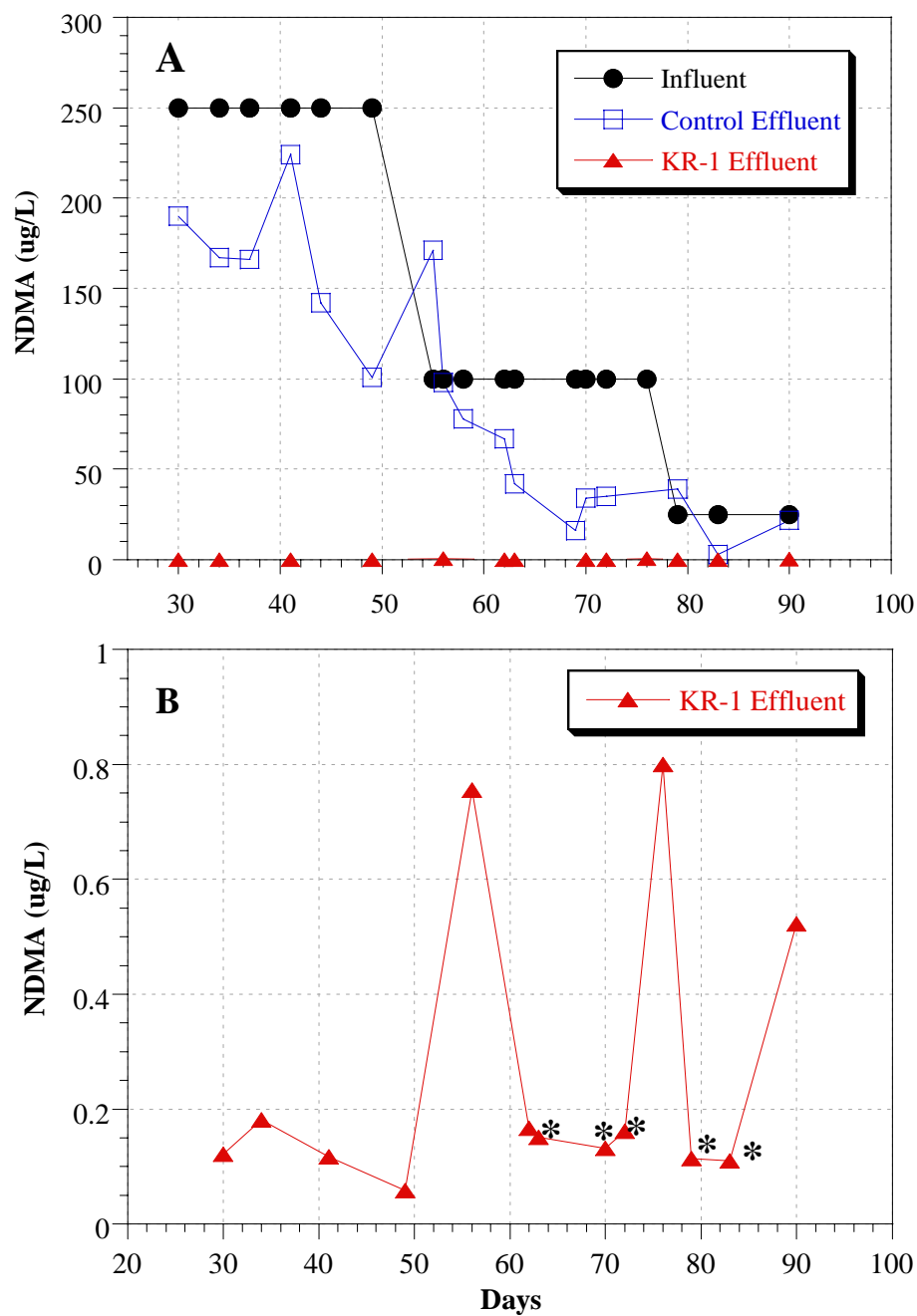


Figure 2. Influent and effluent NDMA concentrations in test and control chemostat reactors (Panel A). Panel B represents effluent levels on a scale from 0 – 1 $\mu\text{g/L}$. Values designated with an * were less than the quantitation limit.



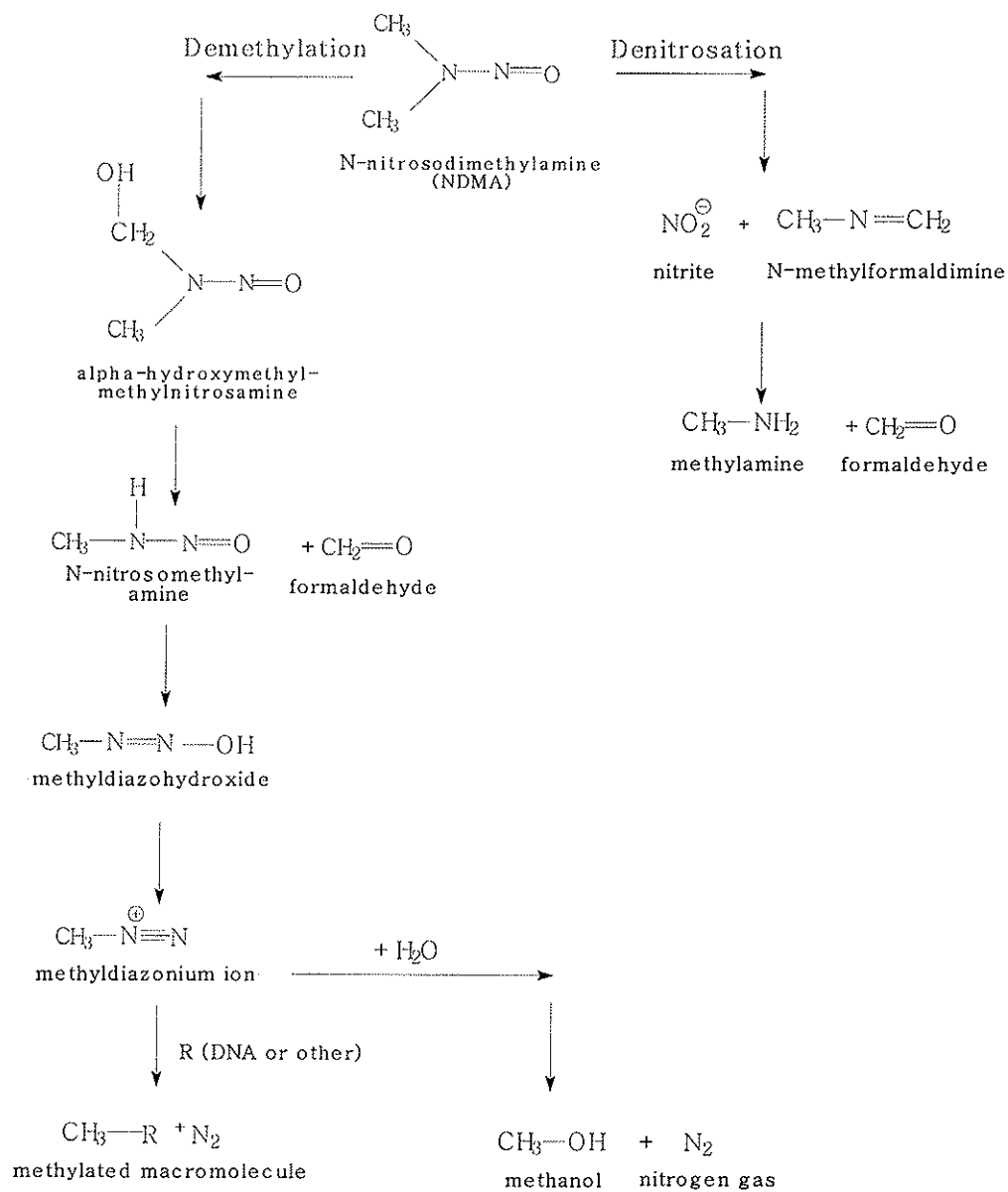
3.0 TECHNICAL APPROACH

The objective of this SERDP project was to evaluate the potential for biological remediation of NDMA in groundwater. The most promising approaches identified in this SERDP project will be developed into field-scale remediation technologies. The specific research tasks completed during the project are given below.

3.1. Determine Pathway(s) of NDMA Biodegradation by Toluene-Oxidizers and Propanotrophs.

The pathways of NDMA biodegradation by the toluene-oxidizer *Pseudomonas mendocina* KR1 and the propanotroph *Rhodococcus ruber* ENV425 were evaluated during the project. The pathways were studied in collaboration with Dr. Jalal Hawari and Dr. Diane Fournier at the NRC Biotechnology Research Institute, Montreal, Canada. The metabolic pathway(s) for NDMA degradation by propane- and toluene-oxidizing bacteria have not been previously reported. However, data from mammalian studies suggest two likely routes of NDMA metabolism by bacteria, denitrosation and demethylation (also called *alpha* hydroxylation) as detailed in Figure 3. These mammalian pathways, which are initiated by Cytochrome P450 enzymes (P450), have been extensively studied due to their capacity to create a highly carcinogenic species (methyldiazonium ion) from NDMA. Similar pathways have also been hypothesized for NDMA degradation by bacteria, including methanotrophs (Yoshinari, 1995), intestinal organisms (Rowland and Grasso, 1975) and undefined organisms from natural soils and waters (Kaplan and Kaplan, 1985). However, few conclusive data are available concerning microbial pathways for NDMA degradation by pure or mixed cultures.

Figure 3. Degradation pathways of NDMA in mammals (modified from WHO, 2002).



3.2. Evaluate NDMA Biodegradation Under Different Electron-Accepting Conditions.

Previous studies provide somewhat confusing and variable conclusions concerning the potential for NDMA biodegradation in environmental samples under aerobic conditions, and there is very limited information concerning NDMA degradation in anaerobic environments. In some instances, extensive mineralization of NDMA has been reported in aerobic samples, whereas in other cases, the nitrosamine appears to be highly resistant to biodegradation. For example, Kaplan and Kaplan (1985) observed extents of NDMA mineralization in lake water ranging from ~ 15% to nearly 80% over a 114-day incubation period. The mineralization increased with declining NDMA concentrations. In addition, rapid and extensive biodegradation of NDMA has been reported in surface soils (Oliver et al., 1979; Yang et al., 2005) and vadose samples (Bradley et al., 2005). In contrast, other studies conducted with various environmental samples, including soils, lake water, and sewage, have shown NDMA to be recalcitrant to biodegradation or only slowly degraded (Tate and Alexander, 1975, 1976; Mallik and Tesfai, 1981). The persistence of NDMA derived originally from 1,1-dimethylhydrazine-based rocket fuel over decades in some groundwater aquifers (e.g., Air Force Site PJKS and Rocky Mountain Arsenal in CO and Aerojet in Sacramento, CA) further supports the conclusion that this molecule can be very recalcitrant under some conditions (Girard, 2000; Gunnison et al., 2000; USEPA, 2004, 2006)).

The environmental data are difficult to explain because there is a general absence of information concerning the mechanism of NDMA metabolism in various environments (e.g., growth substrate, co-metabolism or nitrogen source) and few if any pure cultures have been isolated and studied from natural systems. In addition, it is difficult to compare results and draw general conclusions due to the wide differences in study conditions among research groups. Moreover, with the exception of an early study of intestinal bacteria (Rowland and Grasso, 1975) and a recent paper on vadose soils (Bradley et al., 2005) there is little information concerning the anaerobic degradation of NDMA in natural environments. The objectives of this task were (1) to assess the aerobic biodegradation of NDMA in a series of different environments under the same general study conditions and to assess the most probable mechanisms of loss; (2) to evaluate anaerobic degradation under different electron-accepting regimens; and (3) to attempt to isolate pure cultures or consortia from samples for additional study.

3.3. Evaluate NDMA Treatment in a Laboratory-Scale Bioreactor.

The biodegradation of $\mu\text{g/L}$ concentrations of NDMA was evaluated for ~ 380 days in a propane-fed membrane bioreactor (MBR). Preliminary studies in our laboratory showed that NDMA can be consistently biodegraded in a simple chemostat from 25 – 200 ppb to $< 0.2 \mu\text{g/L}$ using toluene as a primary substrate (Figure 2). For this task, an MBR was constructed at the laboratory scale. The reactor design and operational conditions were selected based upon pure culture studies in Task 1. This reactor was seeded with

Rhodococcus ruber ENV425, and fed propane as a primary substrate. Influent NDMA concentrations ranging from 10 – 100 µg/L were fed to the reactor throughout the study. The joint treatment of chlorinated solvents and NDMA was also examined (see Section 3.4). These studies represent the first time that an MBR has been tested for biological treatment of NDMA.

3.4. Evaluate Influence of TCE and Other Co-Contaminants on NDMA Biodegradation.

Chlorinated solvents, including trichloroethylene (TCE), are often found co-mingled with NDMA at contaminated military and aerospace facilities. The co-occurrence of these contaminants reflects historical joint disposal of solvents and UDMH in open-burn open-detonation areas, landfills, and at other locations. Because TCE is also a target for both toluene-oxidizers and propanotrophs (e.g., Nelson et al., 1986; Wackett et al., 1989), the potential for joint treatment of these co-contaminants by both classes of organisms was tested. Initial studies were conducted with pure cultures in serum bottles to examine the relative degradation rates of TCE and NDMA by the cultures and to determine the extent of competitive inhibition between the co-contaminants. In addition, the contaminants were added jointly to the membrane MBR to evaluate whether they could be treated simultaneously, and also whether TCE impacted NDMA degradation (see Section 3.3) and to aquifer microcosms (see Section 3.5) to evaluate possible treatment options for co-mingled plumes.

3.5. Evaluate Potential for NDMA Treatment using Biostimulation and Bioaugmentation.

The effectiveness of propane and other co-substrates to stimulate naturally-occurring NDMA-degraders was evaluated using aquifer samples collected from three different sites. The effectiveness of bioaugmentation with the propanotroph *Rhodococcus ruber* ENV425 was also examined in aquifer samples and commercial wastewater. For aquifer samples, the mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ was quantified in samples receiving propane, yeast extract, or lactate as cosubstrates. Based on the results of these studies, the extent of NDMA biodegradation was subsequently quantified in large-scale microcosms pre-incubated with propane to stimulate natural propanotrophs. The joint treatment of NDMA and TCE was also evaluated in aquifer samples to determine if both contaminants could be treated via the same co-metabolic approach.

4.0 METHODS, RESULTS, AND TECHNICAL ACCOMPLISHMENTS

The methods, results, and technical accomplishments from this project are detailed in the following sections.

4.1 Pathways of NDMA Biodegradation

The pathways of NDMA biodegradation by the toluene-oxidizer *Pseudomonas mendocina* KR1 and the propanotroph *Rhodococcus ruber* ENV425 were determined during this project. This work has led to two research papers (Fournier et al., 2006; Fournier et al., 2009). The experimental methods, results, and discussion for these studies are provided below.

4.1.1 Pathway of NDMA Degradation by *Pseudomonas mendocina* KR1

Previous studies in our laboratory and others have revealed that two toluene-oxidizers expressing a toluene-4-monooxygenase (T4MO), namely *P. mendocina* KR1 (Whited and Gibson, 1991) and *Pseudomonas* sp. ENVPC5 (McClay et al., 1995) are able to degrade NDMA (Table 1; Streger et al., 2003; Sharp et al., 2005). The involvement of a T4MO enzyme in NDMA degradation was confirmed using a *P. putida* strain transformed with a vector expressing T4MO (Figure 1; Streger et al., 2003; Fournier et al., 2006). In this task, the pathway of NDMA metabolism by *P. mendocina* KR1 was investigated.

4.1.1.1 Materials and Methods: *P. mendocina* KR1

Chemicals

N-nitrodimethylamine and N-nitromethylamine were obtained from Ron Spanggord (SRI International, Menlo Park, CA). [¹⁴C]-NDMA (specific activity of 55 mci/mmol) was purchased from Perkin-Elmer (Boston, MA). NDMA and all other chemicals used in this study were from Sigma-Aldrich and were reagent grade or higher purity.

Analytical methods

NDMA, N-nitrodimethylamine (NTDMA) and N-nitromethylamine (NTMA) were analyzed using the HPLC method previously developed for the quantification of nitro-2,4-diazabutanal (NDAB) (Fournier et al., 2005). Analyses of nitrate (NO₃⁻), nitrite (NO₂⁻), formate (HCOO⁻), formaldehyde (HCHO) and methanol (CH₃OH), were performed as previously described (Hawari et al., 2002). Methylamine and dimethylamine were analyzed as described by Gui et al. (2000). The capture and quantification of ¹⁴CO₂ were performed as described previously (Fournier et al., 2002).

Assay conditions

The degradation pathway of NDMA by toluene-grown *P. mendocina* KR1 was investigated. The bacterium was initially cultured in a nephelo culture flask (Bellco Biotechnology) with basal salts medium (BSM-S; Sharp et al., 2005). Neat toluene (100 μ L per 100 mL culture) was placed in the sidearm tube of the flask. The toluene subsequently volatilizes and dissolves in the culture medium with time to provide organic growth substrate to the strain. The cells were incubated at 30°C and agitated on a rotary shaker at 175 rpm. After growth to late log phase, the cells were harvested and washed twice in mineral salts medium to remove residual toluene. The cells were then added to 6 ml vials containing BSM-S with NDMA (325 μ M) in the absence of a second carbon source. The final absorbance ($A_{600\text{nm}}$) of the culture in the reaction vials was ~ 2.0 . The vials were covered in foil to prevent photolytic degradation of NDMA during incubation.

4.1.1.2 Results and Discussion: *P. mendocina* KR1

Resting cells of KR1 cells completely transformed NDMA (325 μ M) within 27 hr. The degradation of NDMA was concomitant with the rapid and transient production of N-nitrodimethylamine (NTDMA) (Figure 4). The T4MO enzyme appears to add one oxygen atom to convert the nitroso- functional group into a nitro- functional group (Figure 5). The addition of a single oxygen molecule is consistent with other reported reactions of the T4MO enzyme, including the oxidation of toluene to *p*-cresol (Whited and Gibson, 1991) and various alkenes to epoxides (McClay et al., 2000). However, the attack of T4MO on the nitroso group, and the subsequent formation of NTDMA, appears to be a novel metabolic pathway, differing significantly from both the denitrosation and demethylation pathways of NDMA metabolism described for eukaryotes and proposed for various bacteria (see Figure 3). The production of N-nitrodimethylamine was followed by the detection of N-nitromethylamine (NTMA; methylnitramine), formaldehyde and methanol (Figure 4). It is likely that there was a second oxidation of one methyl group to form N-nitrohydroxymethyl-methylamine, which then decomposed to NTMA and formaldehyde. A similar reaction has been proposed for the oxidation of NTDMA by the Cytochrome P450 isozyme CPY2E1 in rat liver microsomes (Frei et al., 1999). It is unclear from the initial studies and the pathway proposed how methanol is generated during this reaction. Previous degradation pathways for NDMA and NTDMA by P450 enzymes, including that described by Frei et al. (1999) appear to generate formaldehyde only. This formaldehyde can then oxidize further to formate as shown in Figure 4 (and was observed in subsequent studies with KR1; see Table 2). It is possible that methanol is formed through enzymatic or abiotic reduction of formaldehyde. Methanol could also be released directly as product from the cleavage of N-nitrohydroxymethyl-methylamine. A further assessment of the potential routes for the generation of methanol during this reaction is presented in Section 4.1.2.

At the end of the 27 hr incubation, 284 and 50 μM of NTMA and methanol were accumulated, respectively. This represents a nearly stoichiometric accumulation of NTMA (325 μM expected), but only approximately 15 % of the anticipated methanol was observed (assuming 325 μM produced) (Figure 4). In a separate study employing radiolabeled NDMA, *P. mendocina* KR1 mineralized less than 5 % of a 270 μM solution containing 500,000 dpm of [^{14}C]-NDMA to $^{14}\text{CO}_2$ during a 27-day incubation (Figure 6). These data suggest that the strain is not utilizing the formaldehyde or methanol generated during NDMA metabolism as an energy source. Slightly higher extents of mineralization were observed if the culture was fed toluene during the incubation period, but extents of mineralization plateaued below 10% after about 27 days.

Figure 4. Biotransformation of NDMA by *P. mendocina* KR1 after growth on toluene.

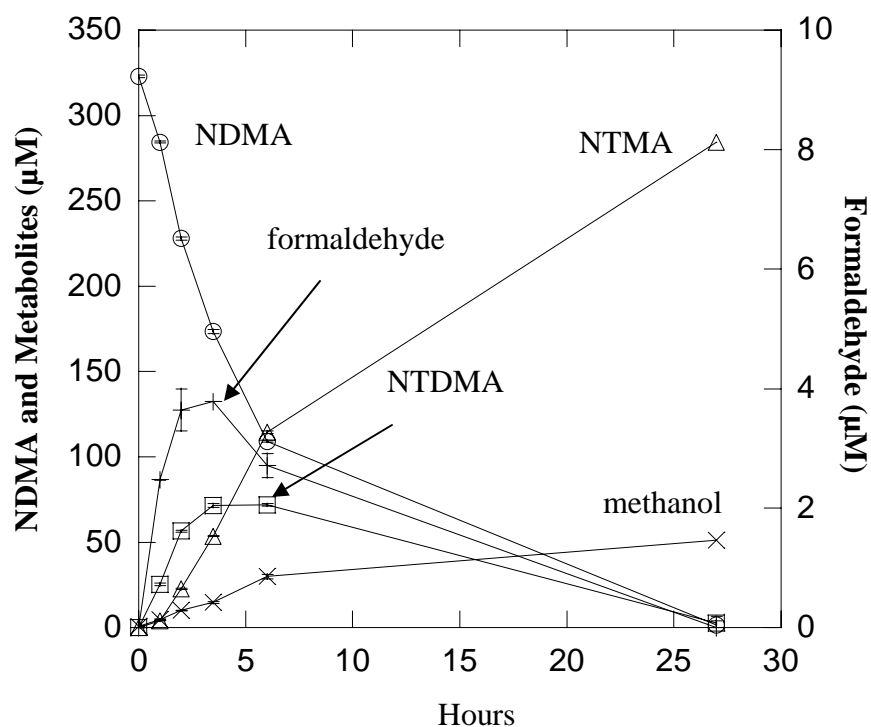


Figure 5. Proposed pathway of NDMA metabolism by *P. mendocina* KR1.

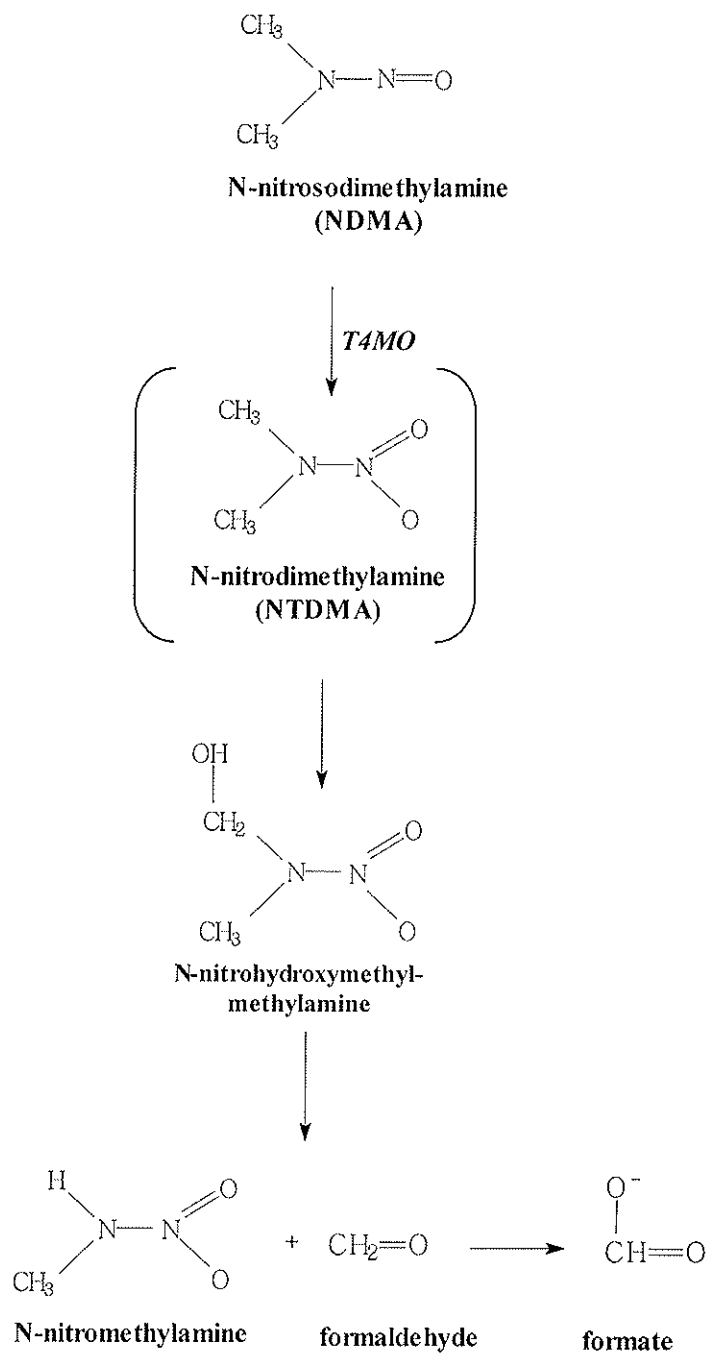


Figure 6. Percent mineralization of NDMA by *P. mendocina* KR1 in the presence or absence of toluene.

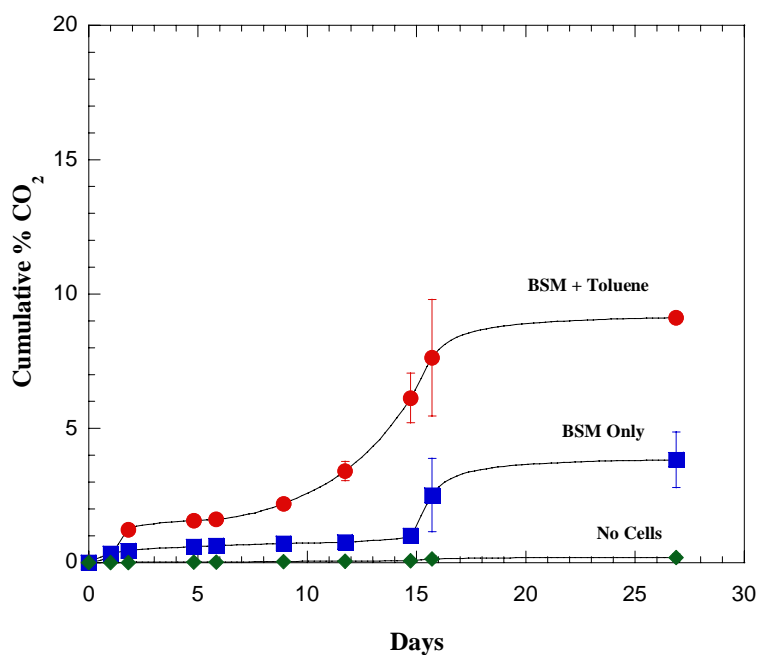


Table 2. Summary of metabolites formed during NDMA biodegradation by *P. mendocina* KR1.

Substrate (μM)	Growth Stage	Transformation Rate ($\mu\text{M h}^{-1}$)	Metabolites (μM)					Stoichiometry (% Recovery)	
			NTDMA	NTMA	HCHO	HCOO ⁻	CH ₃ OH	N	C
NDMA (323)	Early log (OD ₆₀₀ =0.4)	39	Transient (max of 72 μM)	284	Transient (max of 3.8 μM)	n.d.	51	88	60
NDMA (278)	Mid log (A ₆₀₀ =0.9)	132	n.d.	262	Transient (trace)	Transient (max of 20 μM)	22	94	55 ^a
NTDMA (211)	Mid log (A ₆₀₀ =0.9)	193	n.a. ^{b)}	210	Transient (trace)	Transient (max of 26 μM)	n.d. ^{c)}	99	50 ^a

a) The data represent the means from duplicate or triplicate samples, and standard deviations were < 5 % of the mean; b) n.a.: Not applicable; c) n.d.: Not detected

4.1.2 Evaluation of Methanol Production by *P. mendocina* KR1 during NDMA Metabolism

In the previous studies (Section 4.1.1), approximately 92% of the second carbon molecule in NDMA (i.e., that not present in NTMA) was unaccounted for during biotransformation of the nitrosamine by strain KR1. In addition, while the metabolic pathway to NTMA as a terminal end product was observed to be consistent with hypothetical monooxygenase reactions, the pathway used by the bacterium to generate methanol is not readily apparent. Additional studies were performed to (1) confirm the initial reaction pathway to NTMA; (2) attempt to close the reaction mass balance; and (3) confirm that the methanol generated during the metabolic reaction was derived from NDMA.

4.1.2.1 Materials and Methods: Methanol Studies

The chemicals and analytical methods were as described previously in Section 4.1.1.1. Strain KR1 was cultured in a sidearm flask containing BSM-S with neat toluene (100 μ L per 100 mL culture) as the growth substrate in the sidearm tube. The cells were incubated at 30°C and 175 rpm until early-log phase ($OD_{600}=0.37$), at which time the cells were harvested and washed twice in BSM-S. The cells were then added to 6 mL vials containing BSM-S with NDMA (300 μ M) in the absence of a second carbon source. In some studies, radiolabeled NDMA was added and $^{14}CO_2$ was measured as described previously (Fournier et al. 2002). The vials were covered in foil to prevent photolysis of NDMA during incubation. To assess the amount of ^{14}C associated with biomass, the culture was centrifuged, washed, resuspended in BSM-S, then added to liquid scintillation fluid and analyzed for radioactivity.

4.1.2.2 Results and Discussion: Methanol Studies

NDMA degradation by strain KR1 (Figure 7) was more rapid than observed previously (Figure 4). In this study, NDMA was completely transformed within 3.5 hrs whereas in the previous study, > 30% of the added NDMA remained after 6 hrs of incubation (Table 2). Unlike the previous study, NTDMA was not observed as an intermediate in this study. It is likely that this transient intermediate was rapidly transformed to the terminal metabolite NTMA. Presumably, the reaction was faster because the cells (which were harvested in mid-log rather than late-log phase) were expressing more T4MO than in the previous experiment. However, as shown in Figure 7, NTMA was the primary terminal product in this as well as the previous study. The quantity of NTMA recovered accounted for 95% of that expected based on the molar quantity of NDMA in the study. Thus recovery was near complete. Formaldehyde was again a transient intermediate, and methanol was again accumulated to approximately 8% of that expected based on stoichiometry. In this study, formate was also detected as a transient product. Thus, the data from this study generally confirm the previous findings. It should be noted that

neither methylamine nor dimethylamine were detected in this or any subsequent experiments with KR1. Methylamine would be expected if NDMA degradation was proceeding through a denitrosation-type pathway (see Figure 3).

As previously noted, the production of methanol during NDMA oxidation by KR1 is difficult to explain, particularly if this methanol is derived from the reduction of formaldehyde. It is possible that a small amount of the NDMA is metabolized through a *demethylation* reaction, with methanol resulting from the reduction of the methyldiazonium ion, an intermediate that is produced during this pathway (Figure 3). However, prior to examining the hypothesis that NDMA was degraded by a heterogeneous reaction, we felt that it was critical to conclusively prove that the methanol is actually derived from NDMA and not present either as an impurity in the NDMA stock or as a metabolic product from toluene oxidation or another metabolism. Experiments were conducted to rule out these possibilities. In one such study, cells of KR1 were incubated in BSM-S without NDMA, and subsamples were analyzed. Methanol was not detected in this experiment, suggesting that other cell metabolites (e.g., from initial growth on toluene, from autolysis, etc) were not the source of methanol (data not shown).

In a second study to examine methanol formation, a new lot of NDMA was purchased from Sigma-Aldrich ($\geq 99.9\%$ pure) to ensue that a putative impurity in the “old” NDMA stock was not the source of methanol. In experiments with the new stock, NDMA was again transformed into NTMA, giving a molar ratio NTMA/NDMA of 91% (Figure 8). Moreover, similar amounts of methanol were detected as in previous experiments, with a molar ratio (MeOH/NDMA) of 10%. This shows that methanol is not present as or derived from an impurity in the NDMA stock. The experiment also suggests slower NDMA degradation by KR1 results in the formation of less NTMA and more methanol, than when rates are more rapid. This result suggests that two concurrent metabolic pathways for NDMA.

In the next experiment examining the formation of methanol (and the metabolites formaldehyde and formate), the degradation of N-nitrodimethylamine (NTDMA) by KR1 was examined. As previously described, NTDMA is hypothesized to be the initial oxidation product produced during NDMA oxidation by T4MO. In this study, the transformation of NTDMA was completed by strain KR1 within 6 hrs of incubation (Figure 9). The NTDMA was transformed into NTMA, giving a molar ratio (NTMA/NTDMA) of nearly 100%. Traces of formaldehyde were detected at the beginning of the incubation (not shown) and formate was transiently produced. However, methanol was not detected as an intermediate in this study. The experiment was repeated several times (*data not shown*), and methanol was not produced in any study with NTDMA as the parent compound. Indirectly, this experiment shows that methanol originates from NDMA itself. Also, it indicates that NDMA is subject to two different degradation mechanisms: one major route leading to the formation of NTDMA and NTMA (approx. 89-95%) and another minor route (most likely demethylation; Figure 3)

leading to the formation of methanol (7-10%). A summary of results from the different biotransformation studies with strain KR1 is provided in Table 2.

A final experiment was conducted to better quantify the carbon mass balance during NDMA degradation by KR1. In this study, the degradation intermediates were quantified as described in previous experiments, and a duplicate treatment was prepared in which radiolabeled NDMA was used and $^{14}\text{CO}_2$ was measured. NDMA was transformed into NTMA, but with a somewhat lower molar ratio (NTMA/NDMA = 77%) than generally observed in the resting cell assays (Figure 10). NTDMA was not detected. Higher amounts of methanol were produced, giving a molar ratio of 17%. The production of CO_2 reached 3% in 9 days and remained below 4% after 15 days of incubation.

According to the degradation assays performed to date with resting KR1 cells, one of the carbons and both nitrogen molecules present in NDMA are present in NTMA as the terminal degradation product. Approximately 7-17% of the other carbon is present as methanol and approximately 4% is mineralized into CO_2 . Thus, approximately 79-89% of the second carbon present in is not accounted for in our previous studies. One possibility is that the missing carbon is present in cell biomass, either through incorporation for cell growth, or through binding of formaldehyde (and/or the methyldiazonium ion) to proteins, DNA, or other macromolecules. In this case, however, significant carbon was not found associated with the cells of strain KR1. After 5 days of incubation, the radiolabeled carbon was distributed as follows: 1.71% was mineralized to CO_2 , 0.19 % was cell-associated, and 92.67% was present in the supernatant (94.5% recovery). This result differs from that observed in a similar study with the propanotroph *Rhodococcus ruber* ENV425. This strain incorporated ~ 9 % of added ^{14}C -NDMA into cell biomass in the absence of growth substrate, and this percentage increased to as high as 20% if growth substrate was present (see Section 4.1.5). Moreover, the strain mineralized > 60% of the added NDMA to CO_2 under a variety of different conditions. The biological pathway of NDMA metabolism by *P. mendocina* KR1 and other information concerning NDMA degradation by this strain was published in 2006 (Fournier et al., 2006).

Figure 7. Biotransformation of NDMA by *P. mendocina* KR1 after growth on toluene: Experiment 2. Cells were harvested at mid-log phase ($OD_{600}=0.90$). The symbols indicate averages of triplicate experiments and the error bars indicate standard deviations.

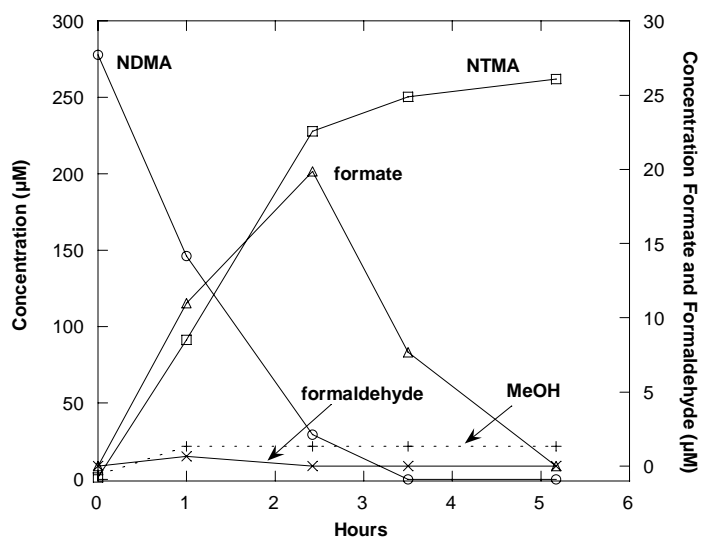


Figure 8. Biotransformation of NDMA by *P. mendocina* KR1 after growth on toluene: Experiment 3. Cells were harvested at mid-log phase ($A_{600\text{ nm}}=0.88$). The symbols indicate averages of triplicate experiments and the error bars indicate standard deviations.

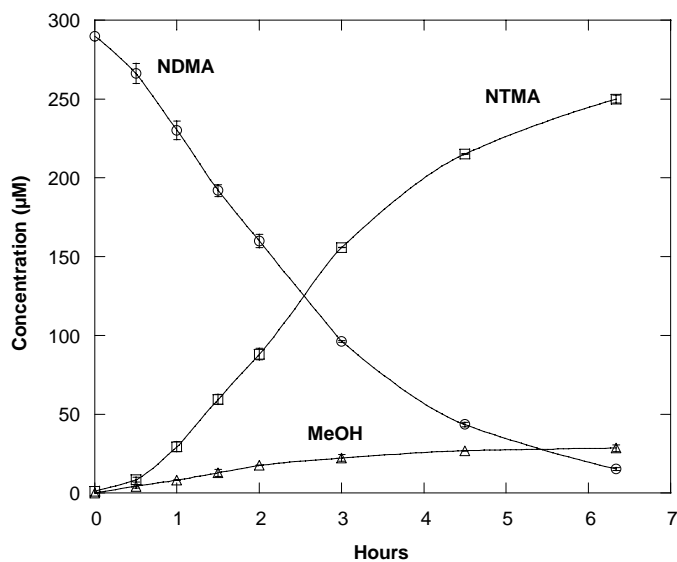


Figure 9. Biotransformation of NTDMA by *P. mendocina* KR1 after growth on toluene. Cells were harvested at mid-log phase (OD_{600 nm} of 0.9). Symbols: NTDMA (O), NTMA (Δ), formate (□). The symbols indicate averages of duplicate experiments and the error bars indicate one standard deviation.

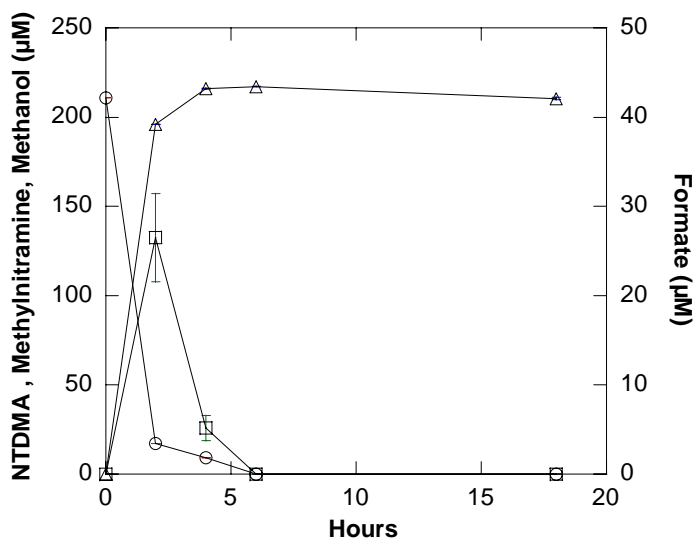
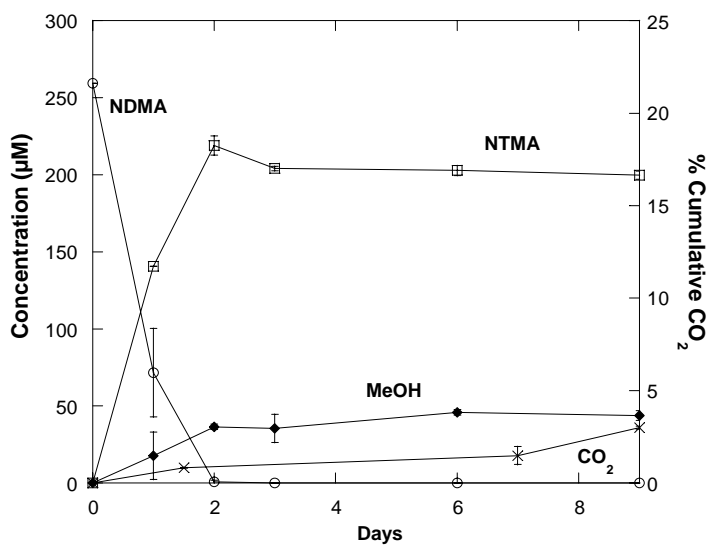


Figure 10. Biotransformation of NDMA and mineralization of ¹⁴C-NDMA to ¹⁴CO₂ by *P. mendocina* KR1 after growth on toluene. The symbols indicate averages of triplicate experiments and the error bars indicate standard deviations.



4.1.3 Confirmation of T4MO as Enzyme Performing Initial Oxidation: $^{18}\text{O}_2$ Incorporation into NTDMA

An experiment was performed to confirm that toluene-4-monooxygenase (T4MO) was the enzyme responsible for the initial oxidation of NDMA by *P. mendocina* KR1. This study consisted of determination of the source of oxygen added to NDMA to produce NTDMA using $^{18}\text{O}_2$ and stable isotope analysis.

4.1.3.1 Materials and Methods: $^{18}\text{O}_2$ Incorporation into NTDMA

P. mendocina KR1 was cultured in a nephelo culture flask (Bellco Biotechnology) with BSM-S. Neat toluene (100 μl per 100 ml culture) was placed in the sidearm tube of the flask as the sole carbon source. The cells were incubated at 30°C and agitated on a rotary shaker at 175 rpm. After growth to mid log phase, the cells were harvested and washed twice in BSM to remove residual toluene. The cells were then added in 0.5 ml volumes to 2-ml vials containing BSM-S with NDMA (325 μM or as specified) in the absence of toluene. A 0.5-ml volume of $^{18}\text{O}_2$ was injected into one set of vials using a gastight syringe. A second set of vials received unlabeled oxygen ($^{16}\text{O}_2$). Two sets of control reactions were prepared, the first containing NDMA without bacterial cells and the second containing bacteria without NDMA. The vials were covered in aluminum foil to prevent photolysis of NDMA and incubated at 30°C (175 rpm). After incubation, the culture was extracted with an equal volume of methylene chloride to remove NDMA and NTDMA for analysis by GC-MS.

An Agilent GC system (Agilent 6890, Agilent Technologies, Palo Alto, CA) coupled to a 5973 quadrupole mass spectrometer (EI) was used to detect ^{18}O in NTDMA. One microliter of methylene chloride extract from the culture study was injected in the splitless mode on a 50 m x 0.2 mm x 0.33 μm HP-5MS capillary column (Agilent Technologies, Palo Alto, CA). The column was heated at 40°C for 2 min then raised to 200°C at a rate of 15°C/min. Helium was used as carrier gas at an average velocity of 28 cm/sec. The injector temperature was set at 150°C and detector interface was maintained at 200°C. Data were collected in the scan mode between 35 and 200 atomic mass units (amu).

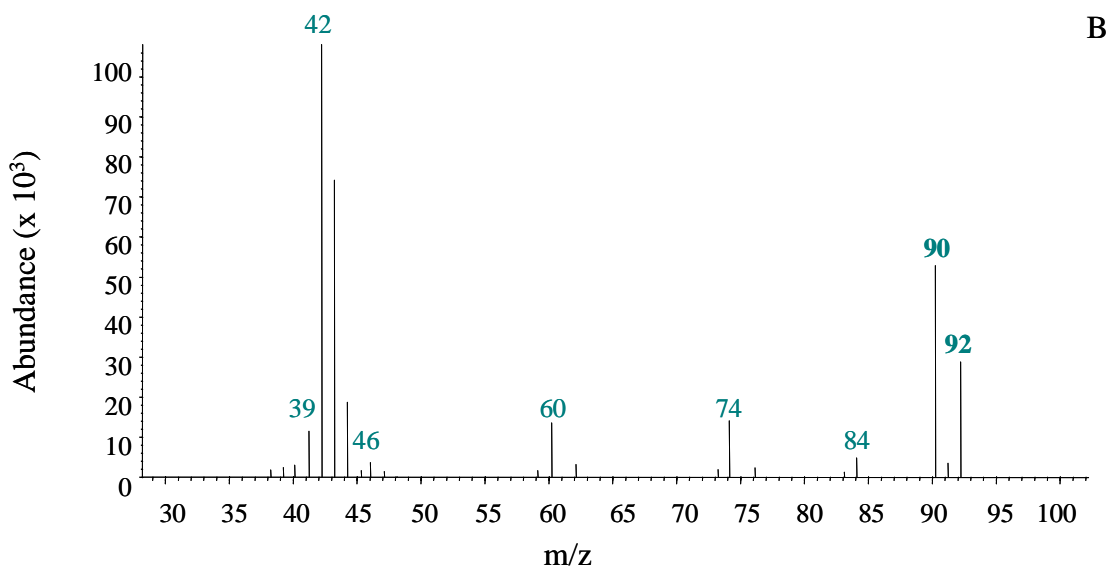
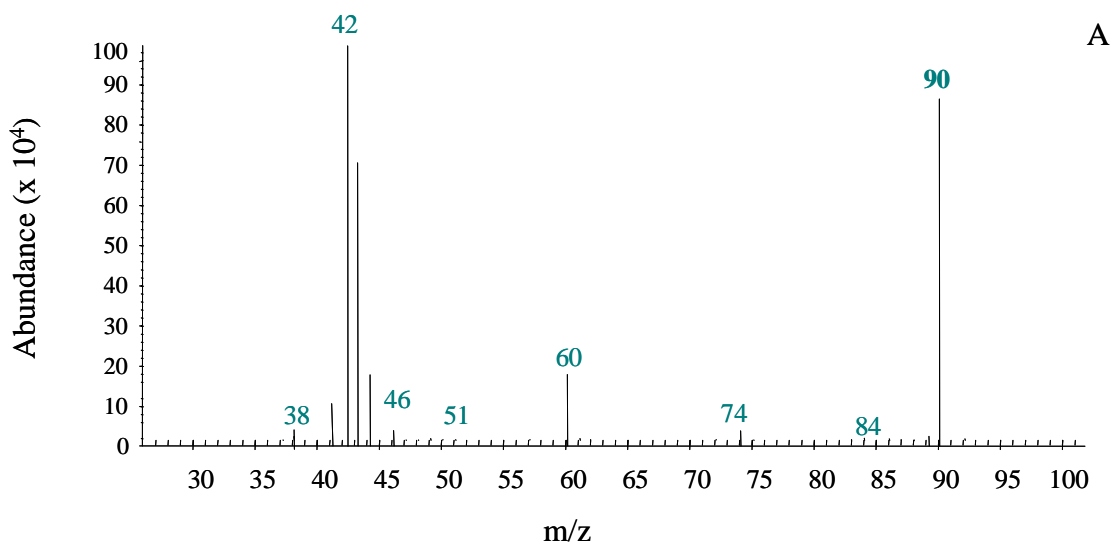
4.1.3.2 Results and Discussion: $^{18}\text{O}_2$ Incorporation into NTDMA

The disappearance of NDMA in vials containing *P. mendocina* KR1 was concomitant with the rapid and transient production of N-nitrodimethylamine (NTDMA) as reported previously. The presence of NTDMA was confirmed by GC-MS (m/z 90 Da) and by comparison with a reference standard (Figure 11A). When the headspace of a vial containing active cells was amended with $^{18}\text{O}_2$, the NTDMA peak showed molecular masses at 90 and 92 Da respectively, corresponding to a mixture of $(\text{CH}_3)_2\text{NN}^{16}\text{O}^{16}\text{O}$ and $(\text{CH}_3)_2\text{NN}^{16}\text{O}^{18}\text{O}$ (Figure 11B). This observation shows that a single oxygen atom originating from air was added to NDMA. The incubation of NDMA with either D_2O or

with H_2^{18}O did not lead to any changes in the mass of NTDMA (i.e., all was present as $(\text{CH}_3)\text{NN}^{16}\text{O}^{16}\text{O}$).

Based on metabolite analysis from previous studies and data from the $^{18}\text{O}_2$ -enriched samples, *P. mendocina* KR1 adds an oxygen atom (O) from O_2 to convert the nitroso functional group in NDMA into the nitro functional group of NTDMA (Figure 5). The addition of a singlet oxygen atom from air to NDMA is consistent with the reactions known to be carried out by T4MO, which is known to attack alkanes, alkenes, and aromatics (McClay et al., 1995, 1996, 2000; Nelson et al, 1986). However the addition of an oxygen atom to the nitroso group of NDMA instead of one of the methyl groups is novel. This reaction differs significantly from both the denitrosation and demethylation pathways of NDMA metabolism catalyzed by P450 enzymes of eukaryotes and proposed for various bacteria (Kaplan and Kaplan, 1985; Tu and Yang, 1985; ASTDR, 1989; Yoshinari and Shafer, 1990). The studies with ^{18}O confirm data generated using the T4MO clone *P. putida* AF, which also suggest that T4MO is the enzyme responsible for the oxidation of NDMA to NTDMA in strain KR1 (see Figure 1).

Figure 11. GC/MS (ES-) spectrum of the metabolite N-nitrodimethylamine (NTDMA) produced during NDMA transformation using resting KR1 cells in the (A) absence or (B) presence of $^{18}\text{O}_2$.



4.1.4 Pathway of NDMA Transformation by *Rhodococcus ruber* ENV425

We evaluated the pathway used by the propanotroph *Rhodococcus ruber* ENV425 to catabolize NDMA. This strain was originally isolated from turf soil using propane as a sole carbon source, and was reported to oxidize methyl tertiary-butyl ether (MTBE) and other gasoline oxygenates (Steffan et al., 1997). Preliminary studies with ENV425 suggest that a propane monooxygenase (PMO) is responsible for its degradation of gasoline oxygenates, but the bacterium is also known to express other oxidative enzymes, including P-450, and alkane monooxygenases (alkB) (McClay et al., unpublished data). The enzyme or enzymes involved in NDMA catabolism by ENV425 are not yet known. However, Sharp et al. (2007) recently showed that the degradation by NDMA by a propane-oxidizing strain of the same genus, *Rhodococcus* sp. RHA1, was mediated by a PMO.

Our data show that the pathway of NDMA degradation by ENV425 differs appreciably from that mediated by the T4MO of *P. mendocina* KR1. Rather, the transformation pathway of NDMA by ENV425 appears to be similar to the denitrosation pathway catalyzed by various P-450 isozymes in mammals, resulting in the production of nitrite, nitrate, formaldehyde, formate, and methylamine (see Figure 3). In addition, a significant fraction of the carbon in the NDMA molecule is released as carbon dioxide by strain ENV425, despite the fact that the bacterium appears incapable of utilizing the molecule for growth.

4.1.4.1 Materials and Methods: NDMA Transformation by *R. ruber* ENV425

Analytical methods

NDMA, NTDMA, NTMA, nitrate (NO_3^-), nitrite (NO_2^-), formate (HCOO^-), formaldehyde (HCHO), methanol (CH_3OH), methylamine and dimethylamine were analyzed as previously reported for strain KR-1 (Fournier et al. 2006 and Section 4.1.1). The capture and quantification of $^{14}\text{CO}_2$ liberated from radiolabeled [^{14}C]-NDMA were also performed as described previously. Nitric oxide (NO) was analyzed by an Apollo 4000 free radical analyzer (WPI, Sarasota, FL) using an ISO-NOP (2.0 mm) NO sensor. Calibration was performed by the chemical generation of NO using a mixture of 0.1 M KI and 0.1 M H_2SO_4 as reducing agent and KNO_2 standard solution (50 μM) as recommended by the manufacturer. The sensitivity of the probe was 1.06 pA/nM. NO in the samples were determined by directly inserting the sensor into the reaction mixture as it was agitated with a magnetic stirrer. The current (pA) generated by the NO present in the sample was recorded. The method detection limit was 4 nM NO.

Growth and assay conditions

To evaluate degradation intermediates, ENV425 was grown in Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ), basal salts medium (BSM-H) (Hareland et al., 1975) with

propane (~ 25% of headspace gas) or basal salts medium (BSM-S; Sharp et al., 2005) with glucose (20 mM) and NDMA (270 μ M). The cells were incubated at 30°C under aerobic conditions and agitated on a rotary shaker at 175 rpm. After growing the culture to late logarithmic phase (OD_{600} of ~ 3.5), the cells were harvested and washed twice in BSM-S without nitrogen or carbon. Cells were then added (OD_{600} of 3.0) to 6-ml vials containing BSM-S and NDMA or NTDMA (135 μ M or as specified). Ammonium phosphate (2 mM) was generally added to the salts solution prior to conducting assays to prevent cell uptake and/or utilization of organic and inorganic N-metabolites (Ecker et al., 1992). Two sets of control reactions were prepared, the first contained NDMA without bacterial cells and the second contained bacteria without NDMA. The vials, covered in aluminum foil to prevent photolysis of NDMA, were incubated at 30°C while shaking at 175 rpm.

Evaluation of NDMA as a sole source of carbon and energy

In one study, ENV425 was tested for growth on NDMA as a sole source of carbon and energy. Duplicate sterile 160-mL serum bottles were prepared in which ENV425 was inoculated into BSM-H supplemented with either 0.3 mM NDMA or 0.3 mM glucose. Controls consisted of ENV425 in BSM-H with no carbon source added. The bottles were periodically opened and a small aliquot was removed for optical density measurement at 600nm (OD_{600}). An additional 0.3 mM of glucose or NDMA was added to the appropriate bottles at select sampling times.

4.1.4.2 Results and Discussion: NDMA Transformation by *R. ruber* ENV425

Initial studies using 14 C-NDMA revealed that strain ENV425 can mineralize significant quantities of NDMA to 14 CO₂ after growth on TSB, glucose, or glucose + NDMA (Figure 12). Greater than 75% of the added radiolabel was recovered as 14 CO₂ in these studies. In a separate study, the strain was also observed to mineralize greater than 60 % of 14 C-NDMA to 14 CO₂ after growth on propane (Figure 13). The presence of propane in the sample appeared to enhance the rate of NDMA biodegradation. Despite the rapid mineralization of NDMA after growth on the aforementioned substrates, our data suggest that ENV425 can not utilize NDMA as a sole source of carbon and energy for growth. When incubated with equimolar quantities of glucose or NDMA, ENV425 grew on glucose, but cell density did not increase with NDMA (Figure 14). This finding was confirmed in subsequent studies in which the ability of the strain to utilize NDMA as source of N was evaluated (see Section 4.1.6).

Based on the initial data revealing that ENV425 rapidly mineralized NDMA, studies were conducted to establish the degradative pathway(s). Washed cells that were initially grown on propane as a sole carbon source degraded NDMA from an initial concentration of 135 μ M to below detection in ~ 2.2 hr (Figure 15). The primary metabolites detected were methylamine (65 μ M), nitrate (39 μ M), nitrite (12 μ M), and

formate (43 μM). Formaldehyde and dimethylamine were also observed at low concentrations, with the former compound reaching a peak concentration of $\sim 6 \mu\text{M}$ after 15 min, then declining thereafter. The concentration of dimethylamine remained below 3 μM . N-nitrodimethylamine (NTDMA) and N-nitromethylamine (NTMA) were not detected. Both of these compounds were observed during NDMA oxidation by the toluene-oxidizer *P. mendocina* KR-1, with the latter compound being the primary terminal metabolite (see pathway in Figure 5; Fournier et al, 2006). Thus, the metabolite data suggest that different degradative pathways are used by the two organisms.

Methylamine and nitrite are expected if NDMA undergoes an initial denitrosation reaction (see Figure 3). However, based on studies in mammalian systems, the initial production of NO would also be expected via the denitrosation but not the α -hydroxylation reaction (Hausmann and Werringloer, 1987). Rapid production of NO was observed from NDMA for cells grown on propane or on glucose (with NDMA added) (Figure 16). These data further support an initial denitrosation pathway for NDMA biotransformation by ENV425. Moreover, the data from glucose-grown cells suggest that at least some of this activity is constitutive or induced by glucose as well as propane.

The main biodegradative pathway of NDMA by ENV425 after growth on either TSB or glucose (+ NDMA) appears to be the same as that for the propane-grown cells. In an initial experiment with glucose-grown ENV425, formate, formaldehyde and nitrite were detected as primary metabolites. Methylamine, dimethylamine and nitrate were not observed. However, ammonium phosphate was not added to the reaction mixture in this study, and it is possible that the aforementioned metabolites were utilized by the bacterium as a source of N (Ecker et al., 1992). When the ammonium phosphate was added, both methylamine and dimethylamine were detected, in quantities similar to those observed for propane-grown cells (Figure 17). In addition, formaldehyde, methylamine, nitrate, and nitrite were detected during metabolism of NDMA by cells cultivated on TSB, suggesting a similar primary reaction mechanism (Figure 18).

In addition to NDMA, ENV425 was observed to biodegrade N-nitrodimethylamine (NTDMA), which is an initial degradation product produced during NDMA oxidation by *P. mendocina* KR1, and is also a suspected carcinogen (see Figure 5). The degradation of NTDMA resulted in the formation of formaldehyde (transient) and NTMA (*data not shown*). NO was not detected. It is likely that NTDMA was degraded by ENV425 via α -hydroxylation of one of the methyl groups, resulting in the decomposition of the molecule to formaldehyde and methylamine, the same mechanism proposed for NTDMA degradation by strain KR1 (Fournier et al., 2006).

The data presented show that the propanotroph ENV425 is capable of rapidly biodegrading NDMA after growth on several different substrates, including propane, TSB, and glucose. The bacterium oxidizes significant quantities of NDMA to CO_2 , but appears unable to utilize the nitrosamine as a sole source of carbon and energy for

growth. The metabolites produced during NDMA degradation by ENV425 after growth on propane include nitric oxide, methylamine, dimethylamine, nitrate, nitrite, formaldehyde, and formate. NTDMA and NTMA, two of the primary metabolites produced during NDMA oxidation by *P. mendocina* KR1 (T4MO), were not detected, indicating that different pathways are utilized by the two bacteria.

Based on the metabolites detected, and the rapid production of NO during NDMA catabolism, ENV425 is hypothesized to degrade NDMA via the denitrosation pathway shown in Figure 19. The metabolites observed during NDMA transformation by ENV425 are the same as those described during denitrosation of NDMA via the cytochrome P450-dependent mixed function oxidase system in mammals (Tu and Yang, 1985; Keefer et al., 1987; OEHHA, 2006). The denitrosation pathway proceeds through the initial enzymatic attack on the nitroso-group of NDMA resulting in the formation of NO and an unstable imine, N-methylformaldimine, which subsequently decomposes to methylamine and formaldehyde (Keefer et al., 1987; OEHHA, 2006). NO is oxidized further to nitrite and then nitrate. Dimethylamine, which was produced in low concentrations from NDMA by ENV425, has also been observed as a minor metabolite in the denitrosation pathway in mammalian systems (Keefer et al., 1987). Thus, all of the key metabolites reported during denitrosation of NDMA in mammals are present during NDMA metabolism by ENV425 after growth on propane and other substrates.

Kaplan and Kaplan (1985) proposed a potential alternate route to the production of nitrite and methylamine during NDMA degradation in environmental samples. They hypothesized that N-nitrosomethylamine (NTMA; an intermediate formed during the α -hydroxylation of NDMA) can degrade to N-hydroxylaminomethylamine, which subsequently decomposes to methylamine and nitrite. We did not observe production of NTMA, which would be expected if this demethylation route were occurring. In addition, NO was produced during NDMA degradation by ENV425. NO would not be expected during the decomposition of N-hydroxylaminomethylamine. Based on these observations (production of NO and absence of N-nitrosomethylamine), we hypothesize that the strain primarily utilizes a denitrosation pathway during NDMA metabolism.

As previously noted, a similar denitrosation reaction in mammals is carried out by P-450 isozyme CYP2E1 (Tu and Yang, 1985). The enzyme responsible for NDMA oxidation in the propanotroph ENV425 is unknown, but is suspected to be a PMO. Preliminary molecular studies with ENV425 reveal that the bacterium contains numerous oxygenases, including PMO, P-450, and alkane monooxygenases (alkB) (McClay et al., unpublished data). Moreover, when grown with propane or glucose + NDMA (but not glucose alone) the PMO, but not the P450 or AlkB genes were induced, suggesting that a PMO is involved in NDMA oxidation. These data support the recent studies of Sharp et al., 2007, who showed that a PMO was responsible for NDMA oxidation in *Rhodococcus* sp. strain RHA1, a bacterium that appears to have similar traits to strain ENV425.

However, additional studies are required with ENV425 to confirm that a PMO is responsible for NDMA degradation.

The key conclusions from the metabolic studies are as follows: (1) the propanotroph *R. ruber* ENV425 and the toluene-oxidizer *P. mendocina* KR1 utilize different metabolic pathways to biodegrade NDMA; (2) ENV425 biodegrades NDMA to the same products (methylamine, nitric oxide, nitrate, nitrite, carbon dioxide) irrespective of initial growth substrate; and (3) the products produced by ENV425 are consistent with a denitrosation pathway of metabolism.

Figure 12. Mineralization of ^{14}C -NDMA into $^{14}\text{CO}_2$ by washed cells of *R. ruber* ENV425 initially cultivated in TSB (●); BSM-S with glucose (◇); or BSM-S with glucose and NDMA. Values from vials with TSB only (no cells) are also shown (□). Values are the means and standard deviations from triplicate samples.

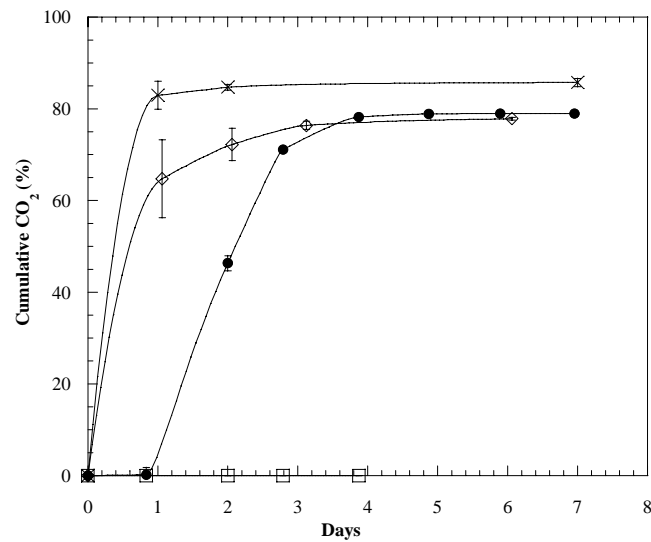


Figure 13. Mineralization of ^{14}C -NDMA into $^{14}\text{CO}_2$ by washed cells of *R. ruber* ENV425 initially cultivated on BSM-H with propane, and then washed and incubated with (●) or without (○) propane. Values from vials with BSM-H only (no cells) are also shown (□). Values are the means and standard deviations from triplicate samples.

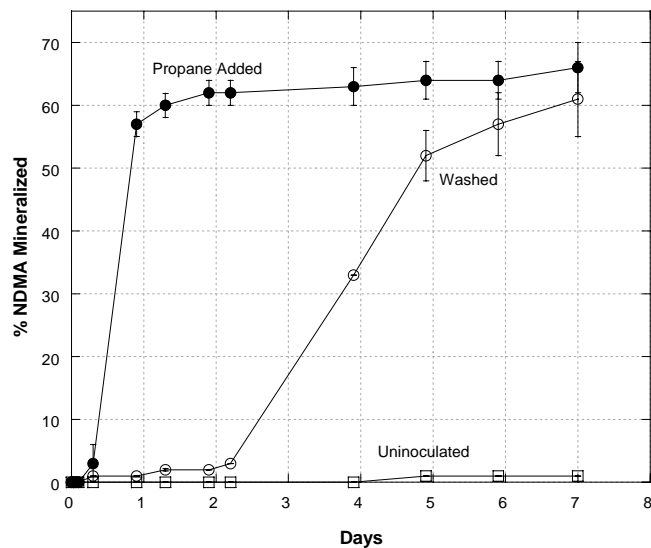


Figure 14. Growth of *R. ruber* ENV425 with glucose (●) or NDMA (○). Uninoculated samples of BSM-H are also shown (□). Values are the means and standard deviations from triplicate samples.

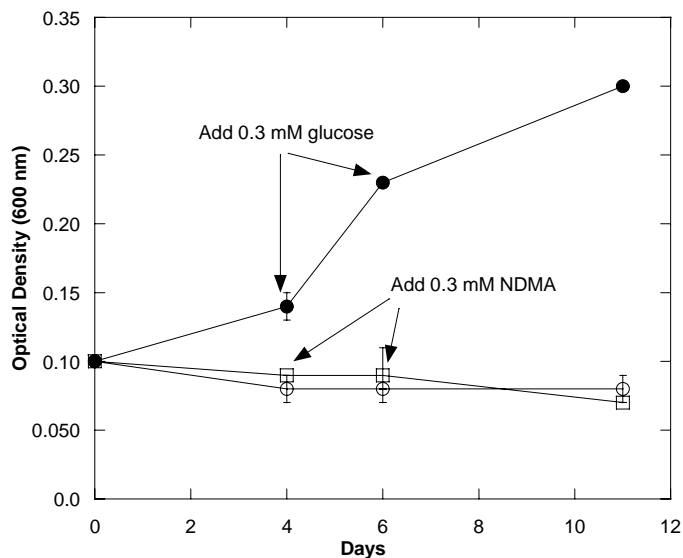


Figure 15. Products observed during the transformation of NDMA by *R. ruber* ENV425 after growth on propane. NTMA or NTDMA were not detected.

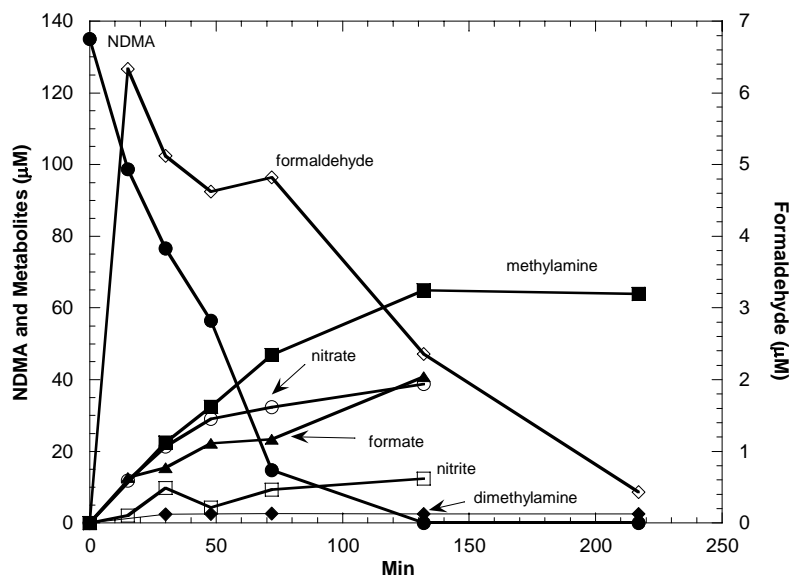


Figure 16. Liberation of nitric oxide (NO) from NDMA by *R. ruber* ENV425 after induction with NDMA (●), NDMA plus propane (x), N-nitrodimethylamine (NTDMA; ◇), and nitrite (□).

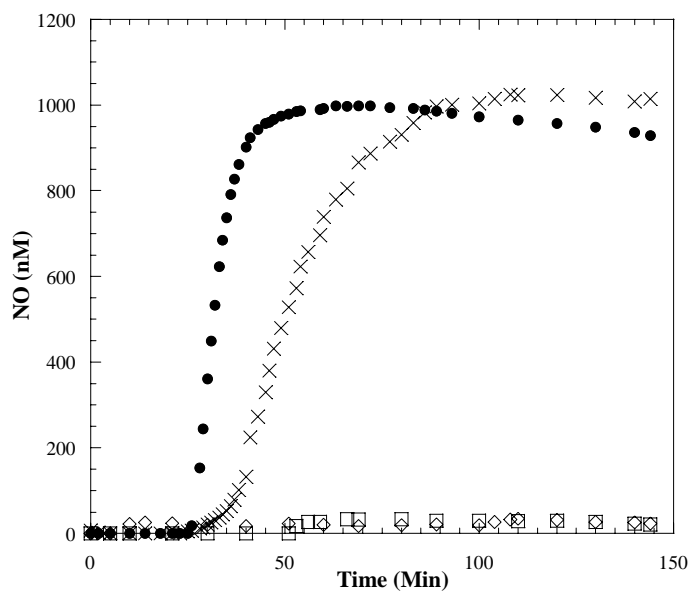


Figure 17. Liberation of methylamine and dimethylamine from NDMA by *R. ruber* ENV425 after growth on glucose + NDMA (closed symbols) or propane (open symbols). Symbols: NDMA (●, ○); methylamine (■, □), dimethylamine (▲, △).

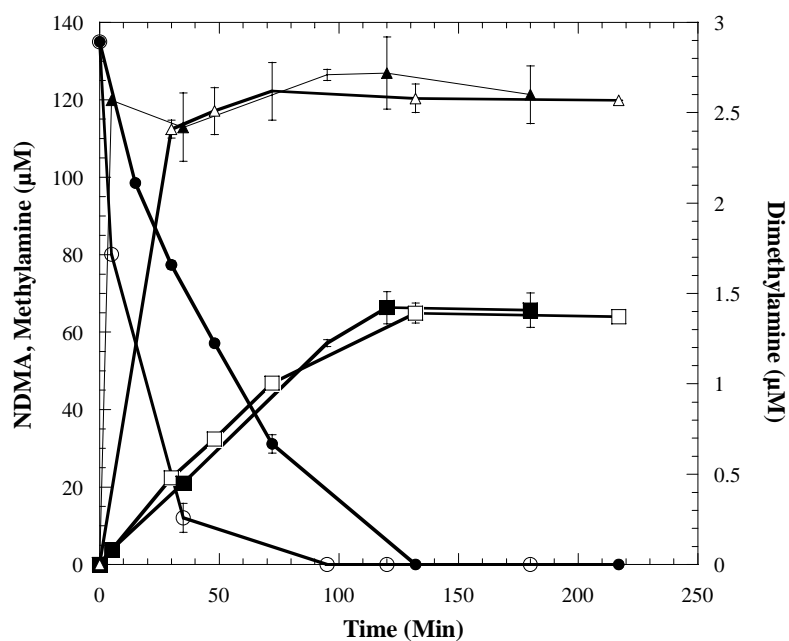


Figure 18. Products observed during the transformation of NDMA by *R. ruber* ENV425 after growth on TSB.

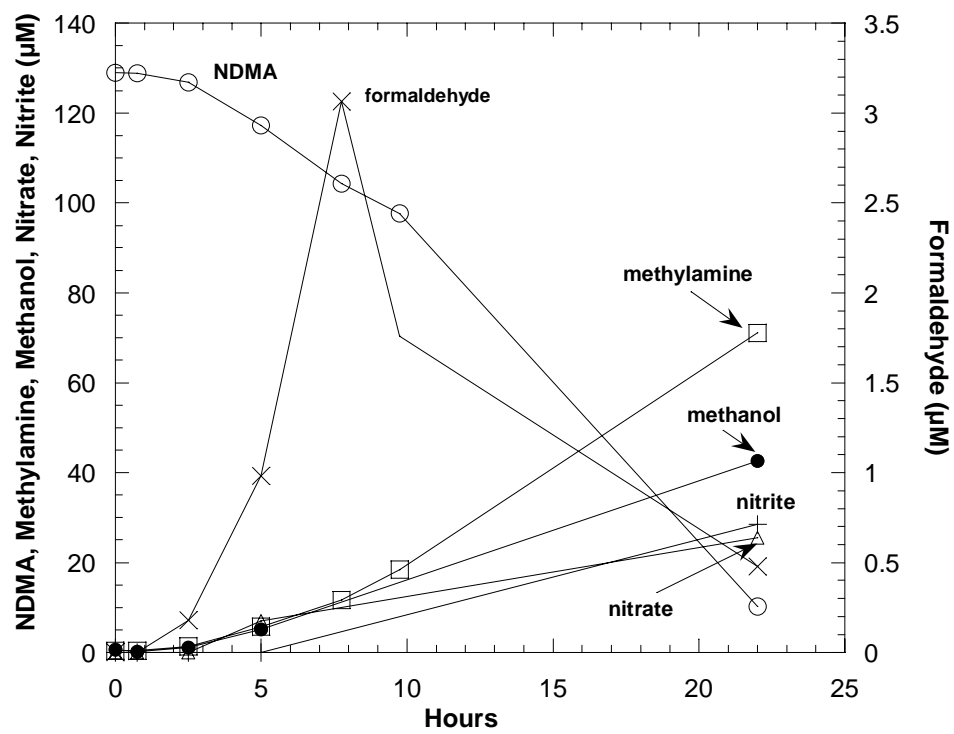
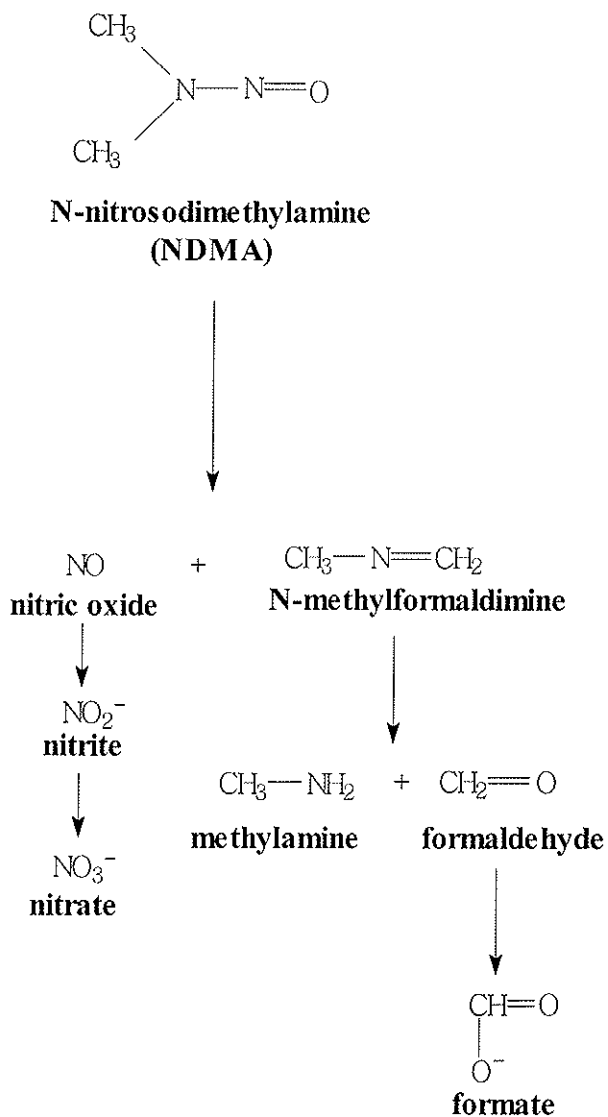


Figure 19. Denitrosation pathway of NDMA metabolism proposed for *R. ruber* ENV425



4.1.5 Mass Balance of ^{14}C During ^{14}C -NDMA Mineralization by *R. ruber* ENV425 Under Different Growth Conditions

4.1.5.1 Materials and Methods: Mass Balance Studies

The mineralization of NDMA by ENV425 following growth of the strain on various PMO-inducing and non-inducing substrates, including propane (30% gas in headspace), glucose (20 mM glucose), and TSB was studied. The cells were initially grown to mid-log phase in a complete mineral salts medium (BSM-H; Hareland et al., 1975) and with one of the substrates. The cells were then washed in BSM-H and added to 160-mL glass serum vials to an OD₆₀₀ of ~ 1.0 in a nitrogen-free BSM (denoted BSM-K) as detailed in Kaplan and Kaplan (1985). The vials received a solution containing non-radiolabeled NDMA mixed with 0.025 μCi [^{14}C]-NDMA (specific activity = 55 mCi/mmol) to achieve a final NDMA concentration of ~ 50 $\mu\text{g/L}$. Duplicate vials also received either glucose (20 mM), TSB, or propane (30% in headspace) to assess the influence of these substrates on active NDMA degradation. Control vials received either BSM-H or TSB but no cells. A small glass test tube containing 1 mL of 0.5 N sodium hydroxide was used as a base trap for $^{14}\text{CO}_2$. Samples were placed on a shaker in the dark at room temperature (28°C) and shaken at ~ 100 rpm. All treatments were performed in duplicate unless otherwise stated.

During incubation, the quantity of $^{14}\text{CO}_2$ was periodically analyzed by scintillation counting of the NaOH solution in each base trap. The solution was replaced each time, and the total $^{14}\text{CO}_2$ was quantified at the end of the study. At the conclusion of the experiments, the cells in each serum bottle were washed and analyzed for biomass-associated radiolabel, and the supernatant present after cell centrifugation was measured as well to quantify soluble radiolabeled products. A mass balance of total radiolabel was calculated for each sample bottle.

4.1.5.2 Results and Discussion: Mass Balance Studies

As previously observed, cells of ENV425 mineralized NDMA after growth on propane, glucose, or in TSB media. When cells were initially grown on TSB, glucose, or propane, then washed and incubated with NDMA (i.e., with no additional carbon added) ~ 8 to 10% of the initial radiolabel was associated with cell biomass (Table 3). There was no appreciable difference in cell-associated ^{14}C among the different initial growth conditions. When the cells were incubated with one of the growth substrates in addition to ^{14}C -NDMA, the cell associated radiolabel was somewhat higher, averaging 14 to 17% of the added ^{14}C . These values are much higher than that observed with strain KR1 (< 0.2% ^{14}C -incorporation), which neither mineralizes NDMA nor appears to incorporate carbon into biomass. The total percentage of ^{14}C recovered in the various treatments with ENV425 generally varied from ~ 80 to 87%, and ~ 105% was recovered from the formaldehyde-killed controls. Thus, although ENV425 does not appear to grow on

NDMA as a sole carbon and energy source, a significant quantity of ^{14}C becomes associated with the cells during biotransformation.

4.1.6 Evaluation of NDMA and NDMA Metabolites as Sources of Nitrogen for ENV425

In metabolic studies, key N-containing degradation intermediates, including methylamine and nitrate (see Section 4.1.4.2), were not observed unless ammonium phosphate (2 mM) was added to the salts solution. It is hypothesized that, in the absence of readily assimilated N, ENV425 utilized one or more of these metabolites from NDMA as a source of N. To evaluate this hypothesis, a study was conducted to assess whether ENV425 was capable of utilizing NDMA and key metabolites of NDMA as sources of N for growth.

4.1.6.1 Materials and Methods: NDMA as a N source for ENV425

ENV425 was initially grown in BSM-H with propane (~ 25% of headspace gas) at 28°C with shaking. After growth to late logarithmic phase (approx. OD_{600} of ~ 3.5), the cells were harvested and washed twice in BSM-H without nitrogen or carbon. Cells were then added at low density (OD_{600} ~ 0.008) in 10-mL volumes to small serum bottles with BSM-H amended with no N source, NDMA, methylamine, nitrate, or ammonium as a sole source of N. The quantity of each compound added was equalized on a molar basis (~ 0.7 mM) based on total N. In addition to these treatments, bottles were prepared with complete BSM-H (with ~ 9.4 mM N from NH_4Cl) as a positive control for cell growth, and with complete BSM-H + NDMA (50 mg/L) to confirm that ENV425 does not use NDMA as a carbon/energy source. Propane (10% of headspace) was added to each bottle (except that with NDMA only) as a carbon/energy source and oxygen (90% of headspace) was added as the electron acceptor. Phosphate was provided in excess in the BSM-H. The bottles were incubated in the dark at room temperature (~ 28°C) on a rotary shaker, and OD_{600} was measured with time to assess growth.

4.1.6.2. Results and Discussion: NDMA as a N source for ENV425

In the presence of 0.7 mM N from ammonium or nitrate, ENV425 grew to an OD_{600} of ~ 0.5 after 7 days, and to an OD_{600} of ~ 1.0 after 21 days (Figure 20). These results confirm that the bacterium is capable of readily using NO_3^- , one of the metabolites from denitrosation of NDMA by ENV425, as a sole N source. Significant growth was also observed over the course of the experiment when methylamine or NDMA were provided as sole sources of N compared to controls receiving no exogenous N. Cell growth with these two compounds was initially slower than when nitrate or ammonium was provided (Figure 20). However, after 21 days, ENV425 grew to cell densities of ~ 0.7 and 1.1 (OD_{600}) with NDMA and methylamine as the sole N sources, respectively. The data suggest that both NDMA and methylamine, can be used by ENV425 as sole sources of N. The apparent lag period suggests that conversion of these species to readily utilized N is

required and that this process may initially limit cell growth. Additional studies are required to better understand N metabolism by this bacterium.

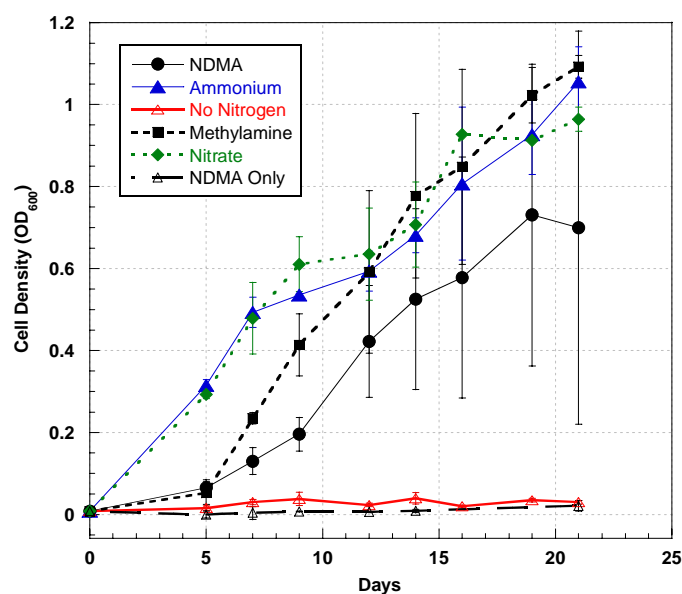
Table 3. Mass balance of ^{14}C during ^{14}C -NDMA mineralization by *R. ruber* ENV425 under different growth conditions.

% Total ^{14}C	Propane-Grown		Glucose-Grown		TSB-Grown		Uninoculated	
	+propane	-propane	+glucose	-glucose	+TSB	-TSB	BSM	TSB
$^{14}\text{CO}_2$	66 ± 4^1	61 ± 6	50 ± 1	69 ± 2	54 ± 7	47 ± 1	1 ± 0	1 ± 0
Supernatant	7 ± 4	9 ± 2	13 ± 7	2 ± 0	11 ± 2	10 ± 2	104 ± 3	103 ± 7
Cells	14 ± 3	8 ± 1	17 ± 3	9 ± 0	16 ± 8	10 ± 0	NA ²	NA
Total	87 ± 11	78 ± 9	80 ± 3	80 ± 3	81 ± 1	67 ± 3	105 ± 3	104 ± 7

¹ Values are the mean + sd from duplicate samples

² NA: Not Applicable – no cells added.

Figure 20. Growth of *R. ruber* ENV425 with propane and NDMA, methylamine, nitrate, or ammonium as sole N sources. Growth with no N and with NDMA only (no propane) is also presented.



4.1.7 Molecular Analysis of ENV425

Based on our previous work with monooxygenase enzymes, we hypothesized that a propane monooxygenase enzyme (PMO) is responsible for NDMA degradation by ENV425. Preliminary studies were undertaken to confirm this hypothesis, as well as to determine how similar in homology the PMO in ENV425 is to the characterized PMO enzymes in other bacterial strains.

4.1.7.1 Materials and Methods: Molecular Analysis of ENV425

Genomic DNA was isolated from ENV425 cells using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Total RNA from bacterial cells was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA) with some modifications to the manufacturer's recommendations. The harvested cells were treated with lysozyme at 37°C for 20 min, after which cell debris was removed by centrifugation at high speed for 10 min. The supernatant was then treated with Trizol and chloroform and washed with propanol. RNA was further cleaned with DNaseI and further purified with the RNeasy Mini kit. PCR-based screening for the presence of monooxygenases capable of propane oxidation was performed using GoTaq Green Master Mix (Promega Inc., Madison, WI) following the manufacturer's guidelines with altered annealing temperatures and extension times as dictated by the DNA sequence being amplified. PCR primer sets designed to amplify the DNA encoding soluble diiron monooxygenases (SDMO; including PMO), AlkB type monooxygenases (AlkB), and P450-type monooxygenases (P450), were used to detect the presence of these enzymes in ENV425 (Heiss-Blanquet et al., 2005; Lisista et al., 2003; Coleman et al., 2006). Real-time quantitative reverse transcriptase PCR (QRT-PCR) was carried out with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Nucleotide sequences were determined with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence was compared with existing sequences in the GenBank database by performing a BLAST search (Altschul et al., 1997).

4.1.7.2 Results and Discussion: Molecular Analysis of ENV425

PCR probing of strain ENV425 chromosomal DNA revealed that representatives of all three monooxygenase sub-families are contained in *R. ruber* ENV425, however, QRT-PCR showed that only SDMO was induced following growth on propane in this organism (Figure 21). Cloning and DNA sequence analysis of the fragment expressed by strain ENV425 following growth on propane identified the gene as a homologue of the PMO present in *Gordonia* sp. Strain TY-5 (Kotani et al., 2003) and its putative homologues in *Rhodococcus* sp. Strain RHA1 (McLeod et al., 2006) and *Mycobacterium smegmatis* MC2 155 (Genbank number CP000480) (Figure 22). Using PCR and inverse PCR we

were able to obtain the sequence for all of the PMO subunits and many of the accessory genes. We found that over the length of the operon, the PMO of ENV425 was 91% homologous to the PMO found in *Rhodococcus* sp strain RHA1, 89% homologous to that of *Mycobacterium smegmatis* MC2 155, and 87% homologous to that of *Gordonia* sp strain TY-5. Additional studies are required to confirm that a PMO is responsible for NDMA degradation after cell growth on propane and other substrates and to assess the potential role of other oxygenase enzymes in NDMA biotransformation by ENV425.

Figure 21. RT-PCR showing the differential expression of the PMO of *R. ruber* ENV425 in response to different growth substrates or amendments. P450 genes identified in the strain were not expressed under the growth conditions used in this experiment.

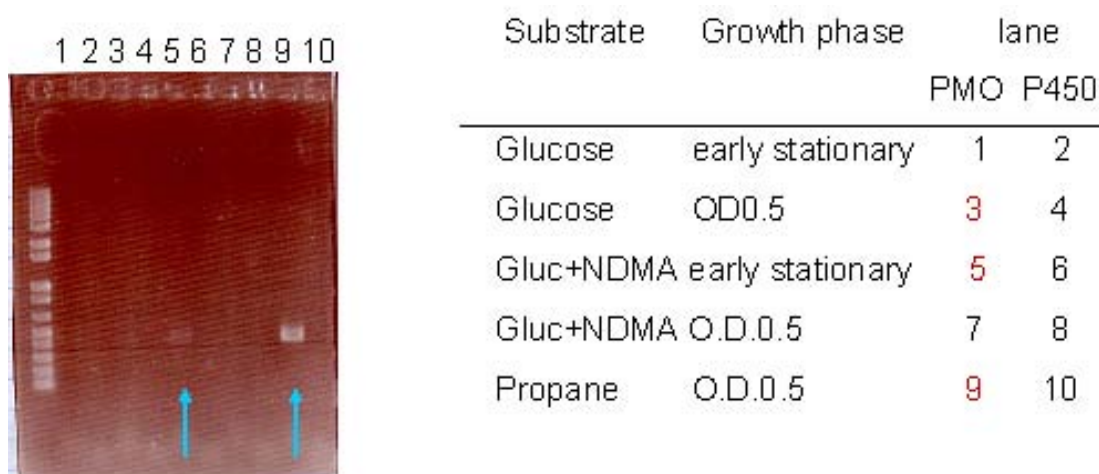
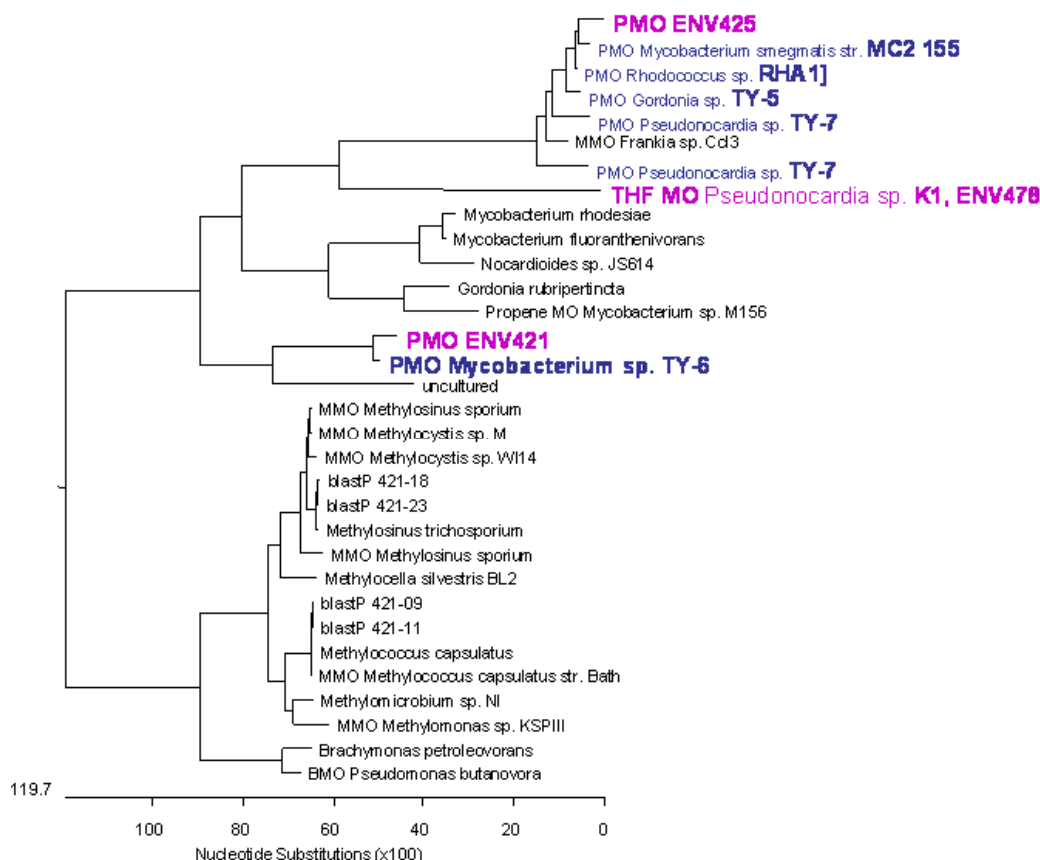


Figure 22. Phylogenetic analysis of diiron containing monooxygenases related to the PMO's investigated in this study.



4.1.8 Conclusions from Pure Culture Studies

The data suggest that *Pseudomonas mendocina* KR1 and *Rhodococcus ruber* ENV425 degrade NDMA by two different pathways. *P. mendocina* KR1, which possesses the T4MO enzyme system, appears to perform an initial addition of singlet oxygen to the nitroso group to form N-nitrodimethylamine (NTDMA), a novel metabolic intermediate for NDMA. The NTDMA is subsequently metabolized to NTMA, methanol, and trace amounts of formaldehyde. Trace mineralization of NDMA carbon to CO₂ was observed, suggesting that this degradation is co-metabolic in nature. The presence of methanol as an end product with NDMA but not NTDMA as the parent substrate also suggests that a second minor pathway of NDMA degradation also occurs in the strain. This pathway is probably the demethylation reaction observed in eukaryotes (see Figure 3). In contrast to strain KR1, the propanotroph *Rhodococcus ruber* ENV425 mineralizes significant quantities (> 60% of ¹⁴C from ¹⁴C-NDMA) of NDMA to CO₂. The bacterium is hypothesized to perform an initial denitrosation reaction that results in the release of NO and n-methylformaldimine, the former of which is then oxidized to NO₂⁻ and then NO₃⁻.

Further biotransformation of the latter compound (n-methylformaldimine) is proposed to result in the formation of methylamine and formaldehyde, the latter of which is oxidized further to formate. Small quantities of methanol and dimethylamine were also detected. The key intermediates produced by *P. mendocina* KR1, namely NTDMA and NTMA were not produced by this strain. Moreover, although *R. ruber* ENV425 can not grow on NDMA as a sole energy and carbon source, the bacterium was observed to use NDMA, as well as NO_3^- and methylamine (key intermediates of NDMA metabolism) as sole sources of N during growth on propane as a carbon and energy source.

4.2 NDMA Biodegradation in Environmental Samples under Differing Electron-Accepting Conditions

A series of studies were conducted to evaluate the potential for aerobic and anaerobic degradation of NDMA by indigenous bacteria from different environments, including soils, sludges, and aquifer samples. The results generated from these experiments are provided in the subsequent sections.

4.2.1 Survey of Aerobic NDMA Biodegradation by Indigenous Bacteria in Different Environments

4.2.1.1 Materials and Methods: Aerobic NDMA Biodegradation

Environmental samples

A variety of environmental samples were collected for evaluation of NDMA biodegradation. Aerobic sewage sludge and sludge from a reaction disc were obtained from a municipal wastewater treatment plant in Hamilton, New Jersey. Surface soil and horse manure were each obtained from farms in New Jersey. Subsurface aquifer samples were obtained from a military facility in central New Jersey (New Jersey site) and Air Force Site PJKS near Denver, Co using direct-push Geoprobe sampling techniques. Groundwater was collected from each site from nearby monitoring wells that had been previously installed. The samples from site PJKS are from an area containing both NDMA and TCE in groundwater, while the New Jersey aquifer samples are from a location contaminated with trace levels of nitramine and nitroaromatic explosives, but from which NDMA is unlikely to occur. In addition to these two sites, aquifer samples were also obtained from a former munitions manufacturing site in southern California, which is now under private ownership. NDMA has been detected in this area at low concentrations. Perchlorate is also present as a contaminant at low ($\sim 300 \mu\text{g/L}$ levels). The cores were obtained during rotosonic drilling activities at the site. As with the other sites, groundwater was obtained from local wells. The aquifer samples were obtained to represent a range of different subsurface conditions, and sampling at two of the sites (New Jersey and southern California) was coordinated with other project activities being

conducted under ESTCP or other DoD remediation activities. All samples were shipped on ice and stored at 4°C until use in microcosm studies.

Aerobic microcosm studies

Initial studies were designed to evaluate the potential for NDMA mineralization by indigenous organisms at each site, so the site samples were added to a mineral salts solution containing inorganic nutrients. The studies were performed in 160-mL glass serum bottles using a filter-sterilized (0.2-µm pore size) nitrogen-free basal salts medium (BSM-K; Kaplan and Kaplan, 1985) modified to include trace metals (Fox et al., 1990) unless otherwise indicated. All bottles and tubes were sterilized via autoclave. Fifty mL of the salts solution and either 0.5 g (solids) or 0.5 mL (liquids) of an environmental sample was added to each serum vial. The vials then received a solution containing non-radiolabeled NDMA (to achieve final concentrations ranging from ~ 10 µg/L to 10 mg/L) mixed with 0.05 µCi [¹⁴C]-NDMA (specific activity = 55 mCi/mmol). A small glass test tube containing 1 mL of 0.5 N sodium hydroxide was used as a base trap for ¹⁴CO₂. Samples were placed on a shaker in the dark at room temperature (~28°C) and shaken at ~ 100 rpm. All treatments were performed in duplicate unless otherwise stated. Co-substrates tested with sewage sludge, farm soil, and manure, and consisted of propane (3% gas in headspace), methane (10% gas in headspace), toluene (headspace saturation via pure toluene in a Durham tube), and acetate (1000 mg/L). Various controls were included in studies, including microcosms without radioactivity (to check for background activity); mineral salts solution receiving ¹⁴C-NDMA but no bacterial inoculum (to evaluate NDMA partitioning to the base trap and abiotic losses); and autoclaved and/or formaldehyde-killed site samples (as killed controls to assess abiotic losses).

Enzyme inhibitor study

Rapid and extensive degradation of NDMA was observed in some microcosms containing environmental samples from various locations (see Section 4.2.1.2). To further elucidate which type of enzymes might be responsible for this degradation, a microcosm study was performed in which several different enzyme inhibitors were tested. Microcosms were set up as described above using a farm soil from New Jersey as an inoculum and nitrogen-free BSM-K medium. Rapid aerobic NDMA mineralization was previously observed in samples from this site. In this study, the following inhibitors were used: chloramphenicol, which inhibits protein synthesis (20 µg/mL); acetylene gas, which inhibits diiron monooxygenases such as T4MO (30% vol/vol in headspace); carbon monoxide, which inhibits cytochrome P450 enzymes (30% vol/vol in headspace); and allyl thiourea (ATU), which inhibits particulate methane monooxygenase (4 mM) (Steffan et al., 1997; Hatzinger et al., 2001; Hamamura et al., 1997). Base traps were used to collect ¹⁴CO₂ as described previously.

Effect of nutrients and trace metals

A study was conducted to assess the influence of inorganic nutrients (nitrogen in particular) and trace metals on the rate of NDMA mineralization in aquifer sediments collected from southern California. Aquifer solids (0.5 g) were incubated with 50 µg/L non-radiolabeled NDMA and 0.05 µCi ^{14}C -NDMA in (1) groundwater only; (2) groundwater with trace metals (Fox et al, 1990); or (3) groundwater with ammonium phosphate (10 g/L $(\text{NH}_4)_2\text{HPO}_4$ + 5 g/L $(\text{NH}_4)\text{H}_2\text{PO}_4$). A sterile-filtered groundwater sample was also prepared as a control. Microcosm base traps were sampled periodically for $^{14}\text{CO}_2$ as described previously. Data was analyzed to determine if the rates of NDMA mineralization differed significantly based on the presence or absence of nutrients and trace metals.

NDMA mineralization kinetics

The kinetics of NDMA mineralization at different concentrations and under different nutrient conditions were quantified. Previous studies have shown an inverse relationship between NDMA degradation rate constants and contaminant concentration. (e.g., Kaplan and Kaplan, 1985). It has also been shown that lower concentration thresholds exist below which bacteria will not degrade a given compound (Alexander, 1994). An experiment was performed to assess the kinetics of NDMA mineralization as a function of concentration using aquifer materials from the New Jersey site. In this study, sediments from New Jersey (0.5 g) were incubated with varying concentrations of NDMA (750 ng/L to 10 mg/L) in sterile 160-mL serum bottles containing the following media: (1) nitrogen-free BSM-K with trace metals, (2) BSM-K with ammonium phosphate, and (3) New Jersey site water. Each bottle received a small tube containing NaOH which was sampled periodically and analyzed for $^{14}\text{CO}_2$ as described below.

Sample collection and analysis

Bottles were sampled by removing the total volume of base from the base trap to a glass scintillation vial. The base trap was then refilled with fresh base. A 10 mL volume of OptiPhase Hi-Safe 3 scintillation cocktail (Perkin Elmer, Loughborough, England) was added to each vial. The vials were then mixed by which was vortex and the radioactivity was counted using a Rackbeta Model 1209 scintillation counter (Pharmacia LKB Nuclear, Gaithersburg, MD) programmed for analysis of ^{14}C . Each vial was counted for 1 min. All headspace gases (i.e. methane and propane) were replenished after sampling, if applicable.

4.2.1.2 Results and Discussion: Aerobic NDMA Biodegradation

Aerobic microcosm studies

At an initial concentration of 50 µg/L, rapid and extensive NDMA mineralization was observed in microcosms prepared from sludge, farm soil, manure, pond sediment, and aquifer solids from New Jersey in the absence of any added cosubstrate (Figure 23). In most cases > 60 % ¹⁴C-NDMA was collected as ¹⁴CO₂ within 20 days. In contrast, little NDMA mineralization (< 10 % in 20 days) was detected in aquifer samples obtained from Air Force Site PJKS. Overall, these initial data suggest that microorganisms capable of mineralizing NDMA are broadly distributed in the environment, although the potential for NDMA degradation in groundwater aquifers appears to be highly site-specific.

The addition of propane, methane, and acetate slightly reduced extents of NDMA mineralization in microcosms prepared from farm soil and sewage sludge, but the effects were not particularly significant, with > 55% mineralized in each case. The farm soil data are shown in Figure 24. Interestingly, toluene completely inhibited NDMA mineralization in these samples. Because the compound was fed through a hydrocarbon feeder (i.e., continuous feed by volatilization into headspace), the effect may merely represent cell toxicity. However, toluene did not inhibit NDMA mineralization in samples prepared from horse manure under identical conditions (Figure 25). Thus, the possibility exists that toluene-oxidizers were performing NDMA degradation in the soil and sludges (but not the manure) and that this activity was inhibited by the presence of toluene.

Follow-up experiments were performed to evaluate whether the observed NDMA mineralization in samples not receiving any organic substrate is co-metabolic (i.e., the cells are growing on available carbon in the samples and metabolizing NDMA fortuitously) or linked to microbial growth (i.e., NDMA is a source of microbial carbon and/or energy). In one study, small aliquots of slurry were transferred from microcosms prepared from New Jersey aquifer materials and farm soil (in which > 60 % mineralization had been observed) to fresh microcosms with 50 µg/L ¹⁴C-NDMA. Interestingly, NDMA mineralization in both sets of samples reached a plateau of < 20 % (compared to > 60 % in the initial study). After 18 days, selected bottles were treated with a filter-sterile extract from the farm soil or the aquifer solids, respectively, as sources of native organic carbon. The mineralization of NDMA in the microcosm receiving the farm soil extract increased markedly, reaching > 60 % in 5 days, while that in the microcosm receiving New Jersey aquifer solid extract remained unchanged (Figures 26a and 26b, respectively). The data from the farm soil study suggest that NDMA mineralization in this environment is co-metabolic and requires available carbon. The data from the New Jersey samples also suggest a co-metabolic process, although, in this case, the addition of organic carbon caused no measurable stimulation in degradation activity. Microbial enrichments were prepared from the samples showing high

degradation activity, but no strains could be isolated that utilized NDMA as a sole growth substrate over several months and in various mineral media +/- exogenous nitrogen (see Section 4.3.1).

Figure 23. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from different environmental samples.

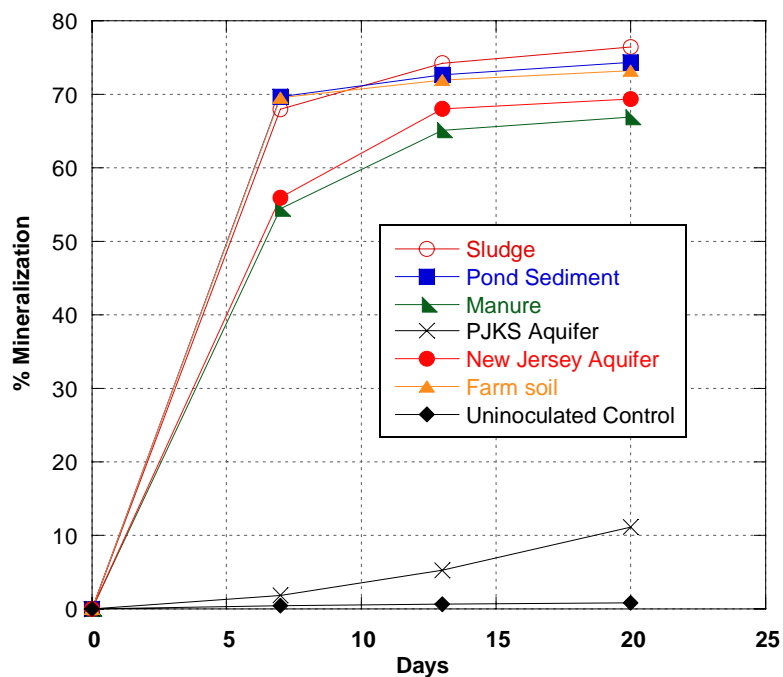


Figure 24. Influence of organic substrates on the mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from farm soil.

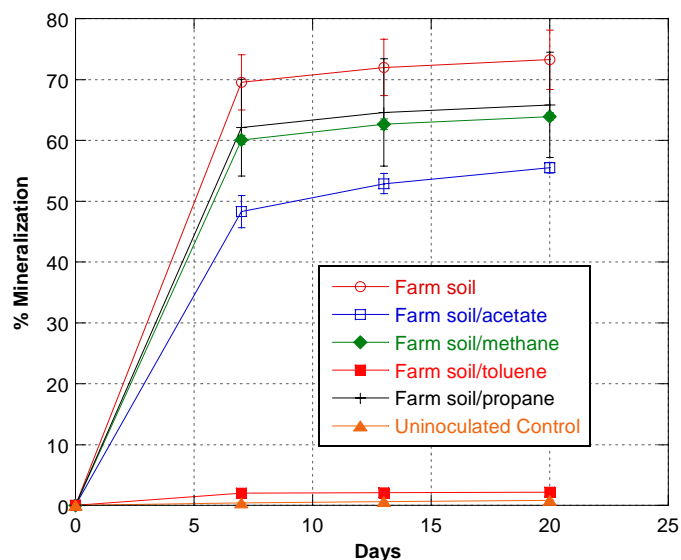


Figure 25. Influence of organic substrates on the mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from horse manure.

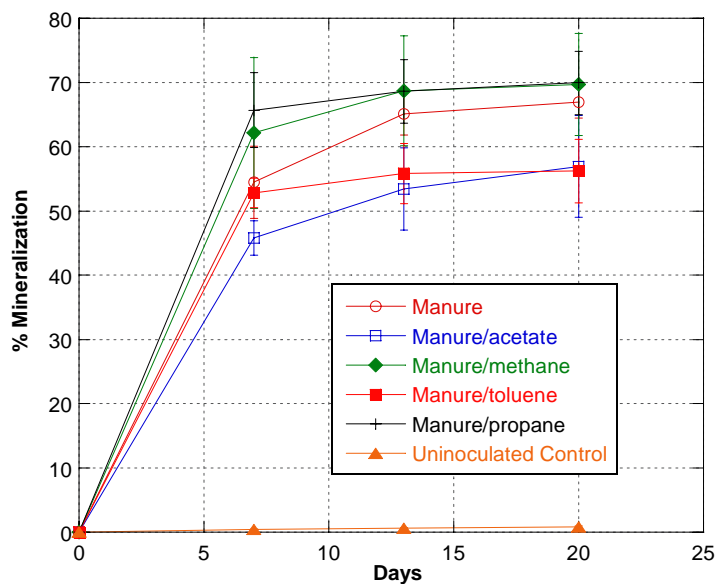


Figure 26a. Influence of sterile soil extract on the mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from farm soil.

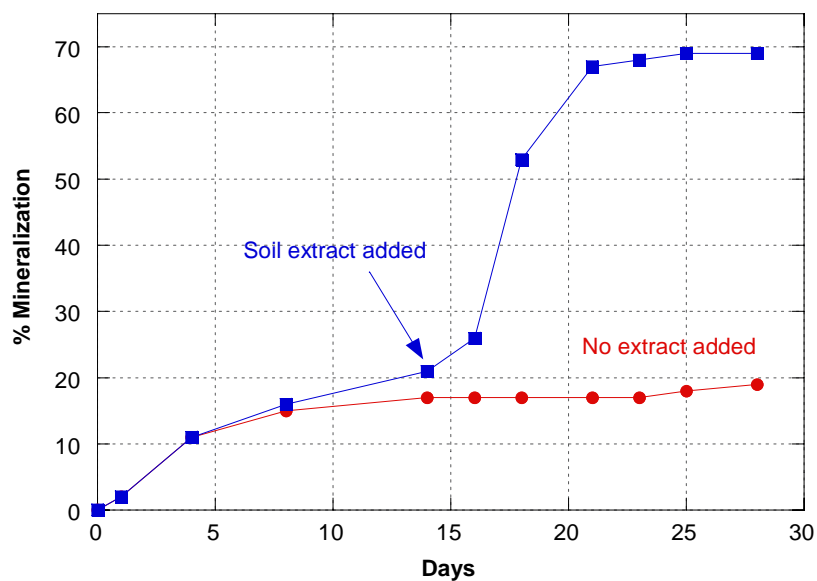
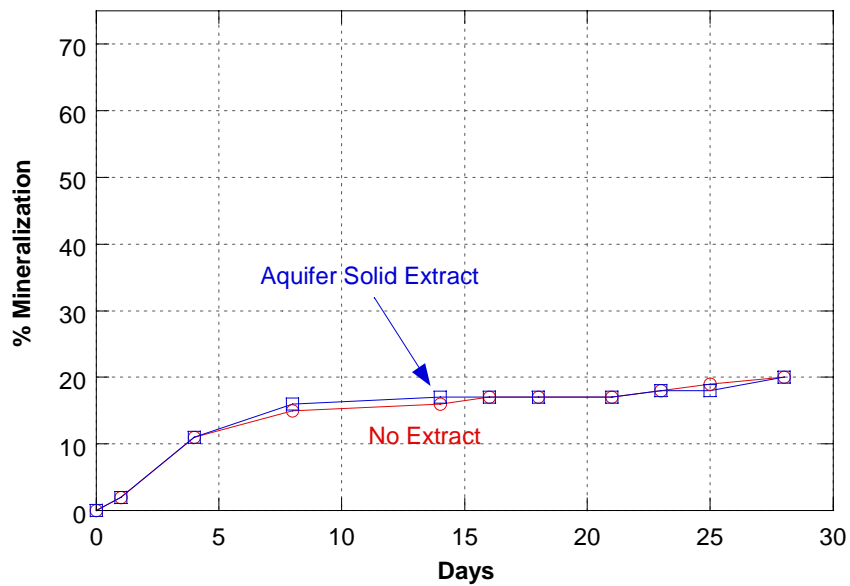


Figure 26b. Influence of sterile soil extract on the mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from New Jersey aquifer solids.



Enzyme inhibitor study

When different enzyme inhibitors were added to bottles amended with farm soil, the rate of NDMA degradation decreased in all samples relative to treatments without added inhibitors (Figure 27). The greatest decrease in the NDMA degradation rate was observed in microcosms to which carbon monoxide was added, indicating that Cytochrome P450 enzymes may play an important role in the observed NDMA mineralization in this environment. However, the fact that the rate of mineralization was reduced to some extent with each of the inhibitors, indicates that a number of different oxygenase enzymes may contribute to NDMA mineralization in natural environments. It is likely that some of the inhibitors biodegraded with time and/or adsorbed to the soil matrix during the study; the actual effect may be greater than reported for some of these compounds

Effect of nutrients and trace metals

The influence of nutrients and trace metals on NDMA mineralization in aquifer samples from southern California was tested. Although there was some variability in the study, the most rapid NDMA mineralization occurred in samples receiving sediment and site groundwater in the absence of added nutrients or trace metals (Figure 28). The data, particularly the slower rates in the nutrient amended samples, suggest that NDMA may be used as a nitrogen source in this environment. It is likely that the trace metal solution added also have had some contaminating nitrogen – perhaps enough to reduce mineralization rates. We previously observed that the propanotroph *R. ruber* ENV425 is capable of using NDMA for N in the absence of other available sources, which confirms that some bacteria are capable of this process (see Figure 20). It should be noted in these studies that NDMA is added at very low concentrations to begin with (50 µg/L in this case), thus small quantities of amendments can make differences not normally expected in biodegradation studies, where contaminants are typically present at mg/L concentrations or higher. However, such low concentrations of NDMA are typical of most contaminated groundwater environments where NDMA biodegradation (and possibly bioremediation) will occur.

Kinetics of NDMA mineralization with New Jersey aquifer solids

A plot of the mineralization curves (% degraded vs. time) of NDMA over concentrations ranging from 0.75 µg/L to 10 mg/L in nitrogen-free basal salts medium is presented in Figure 29. Trace metals were present in this treatment, but macronutrients (N and P) were not. Degradation of 750 ng/L, 10 µg/L, and 100 µg/L NDMA occurred rapidly with no apparent lag, with over 60% of the added NDMA was mineralized within 13 days. As the concentration of NDMA increased to 1 and then 10 mg/L, the mineralization rate (on a % basis) slowed, and only 40 % of the added NDMA was degraded over the course of the study when 10 mg/L was added. This same general pattern was seen when

macronutrients (nitrogen and phosphorus) were added to samples, except that the addition of nutrients caused rates and extents of NDMA mineralization in the microcosms receiving 1 and 10 mg/L NDMA to be appreciably higher than for the treatment without nutrients. When groundwater only was added, mineralization rates were much slower than in the other treatments, and percents of NDMA mineralization remained below 35% at all initial NDMA concentrations (Figure 30).

The maximum rates of NDMA mineralization on a mass basis were calculated by linear regression using the points from the steepest slope of each mineralization curve. These rates are presented as a function of initial NDMA concentration on a log-log plot in Figure 31. The rates of NDMA mineralization ($\mu\text{g/L/day}$) are linear with concentration in the samples receiving BSM + macronutrients (N and P) and in those receiving site groundwater only. This observed linearity suggests that kinetics of NDMA mineralization are approximately first-order in each case. However, although linear with concentration, the actual rates of NDMA mineralization are approximately 10-fold less in samples receiving groundwater than in those with BSM + nutrients. In the treatment receiving BSM but no nutrients, rates are similar to those in the BSM + nutrients at lower NDMA concentrations, but these rates fall significantly as the NDMA concentrations increase, mirroring those in the groundwater samples at the 10 mg/L initial concentration. A plot of the estimated rate constant (day^{-1}) for each treatment vs. the initial concentration is given in Figure 32. This plot shows that, for microcosms receiving aquifer solids with groundwater and to a lesser extent, the nutrient-amended aquifer microcosms, the rate constants remain reasonably constant as NDMA concentrations increase. Conversely, for the samples receiving BSM without nutrients, rate constants decline appreciably as concentration increases.

Overall, the data from this study indicate that, if there is a lower threshold for NDMA degradation for indigenous microorganisms in these samples, that threshold is below 750 ng/L. Mineralization was observed in all treatments at this concentration, which was the lowest tested. These results agree with those of Kaplan and Kaplan (1985), who showed significant NDMA mineralization in lake water samples at levels less than 30 ng/L. The data also suggest that rates of NDMA mineralization are limited in these samples by inorganic constituents. At low concentrations, these constituents may be trace metals, as the degradation rates in samples receiving BSM without nitrogen or phosphorus (but with trace metals) were appreciably higher than in those with groundwater only. As NDMA concentrations become higher, rates appear to be limited by the availability of nitrogen and/or phosphorus. This theory explains the difference in rates between the treatments receiving BSM with or without these macronutrients, which was appreciable at higher NDMA concentrations. It is unclear from the data whether NDMA was utilized as a growth substrate or a carbon source (exogenous carbon was not added) or whether the degradation was purely co-metabolic, but it appears that NDMA was not used primarily as a nitrogen source, at least in the samples receiving BSM.

It should be noted that the data with the New Jersey samples differ appreciably from those in a study with samples from the southern California site, where the addition of nutrients or trace metals slowed degradation rates. The study with the southern California samples was conducted at only one concentration of NDMA, but the data suggest that different degradation mechanisms may be at work in different environments and that inorganic nutrients and/or trace metals may be a key factor in this process.

Figure 27. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms receiving farm soil and various inhibitors of oxidative metabolism.

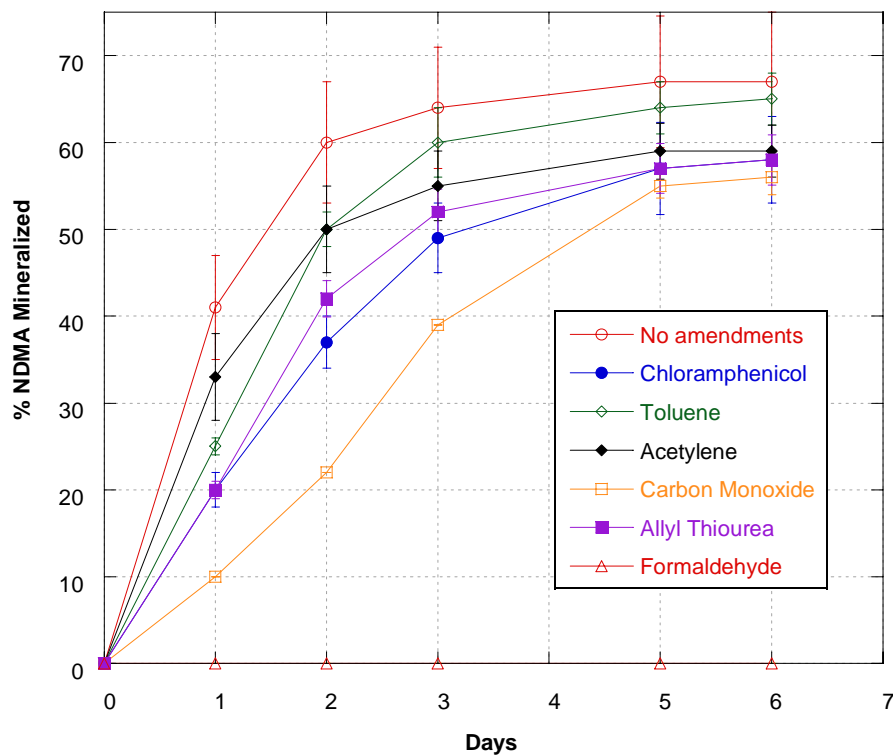


Figure 28. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from southern California aquifer sediments and groundwater alone (GW) with inorganic nutrients (GW + nutrients) or trace metals (GW + Trace metals).

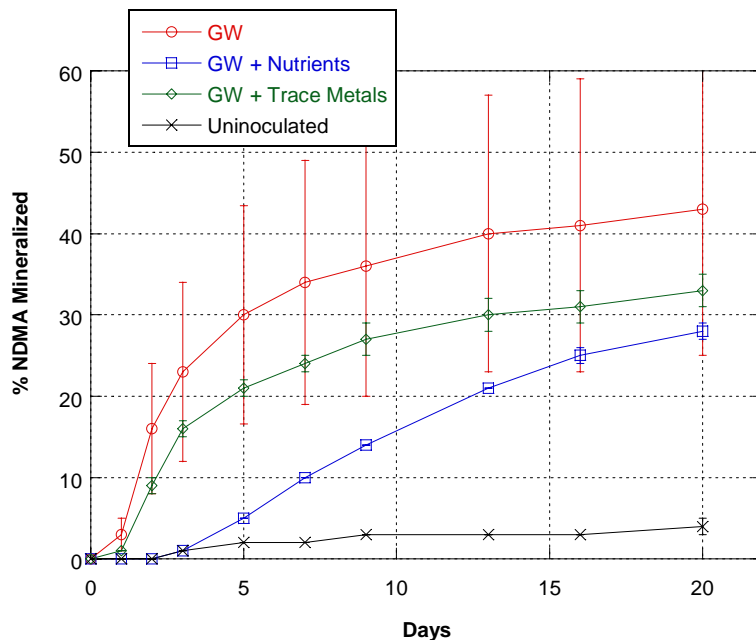


Figure 29. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from New Jersey aquifer sediments and BSM-K (no nitrogen added).

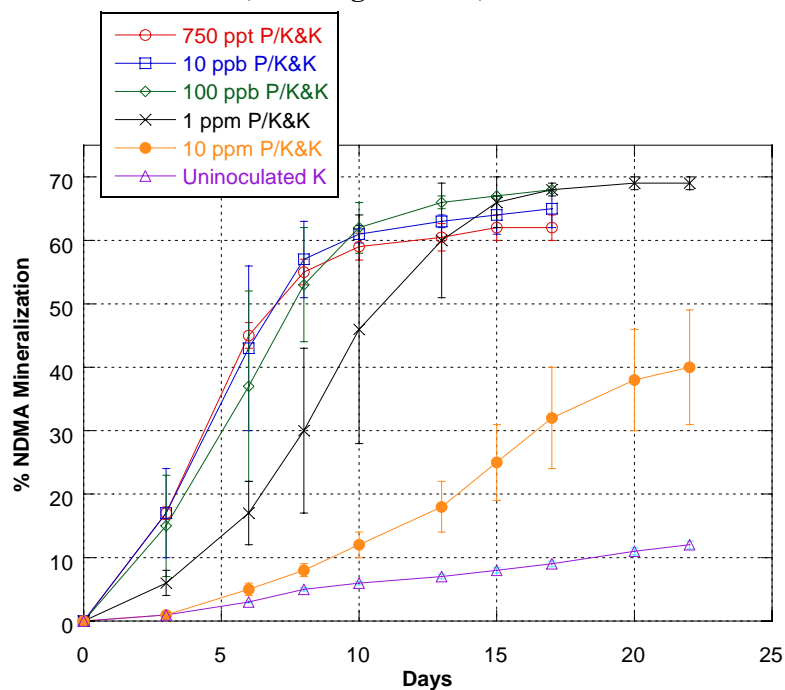


Figure 30. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from New Jersey aquifer sediments and groundwater (no nitrogen added).

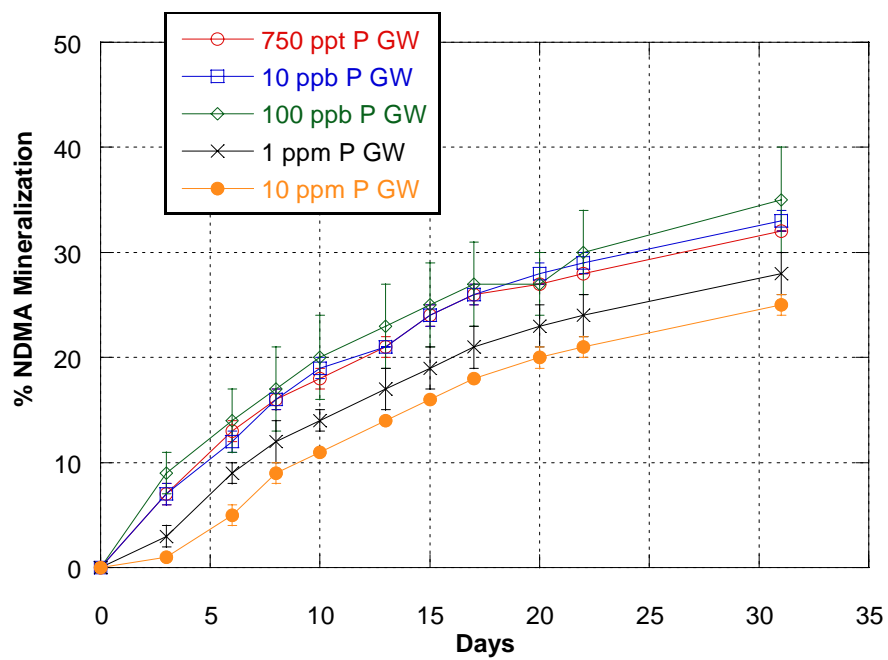


Figure 31. Comparison of Mineralization Rates of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from New Jersey aquifer sediments and BSM-K without N, BSM-K with nitrogen, or groundwater. Values are plotted on a log-log scale.

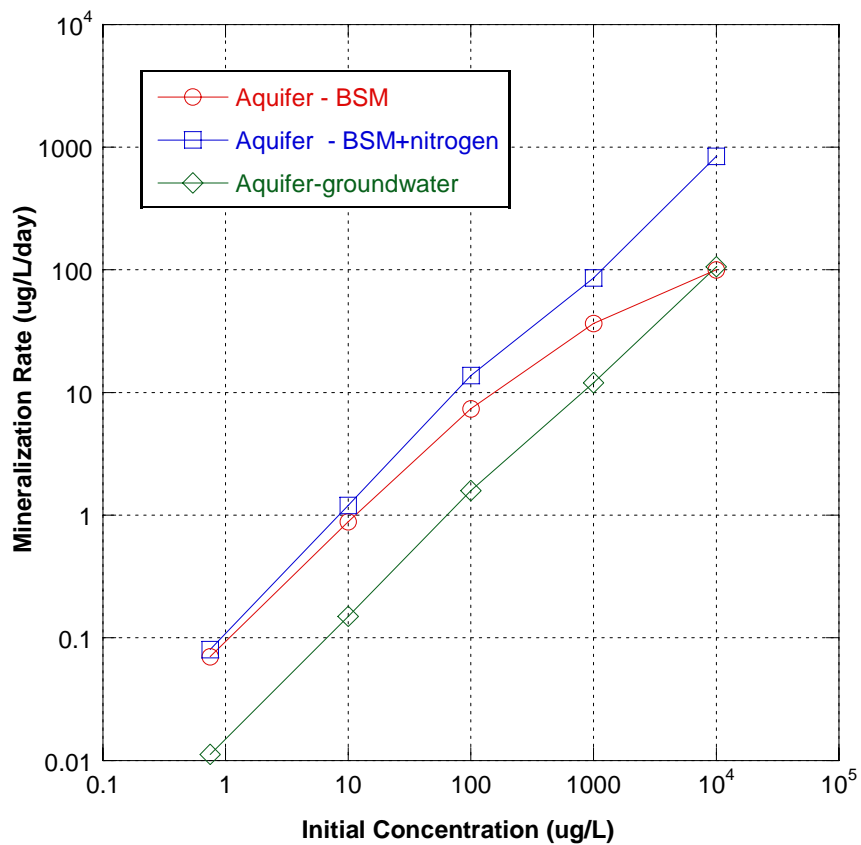
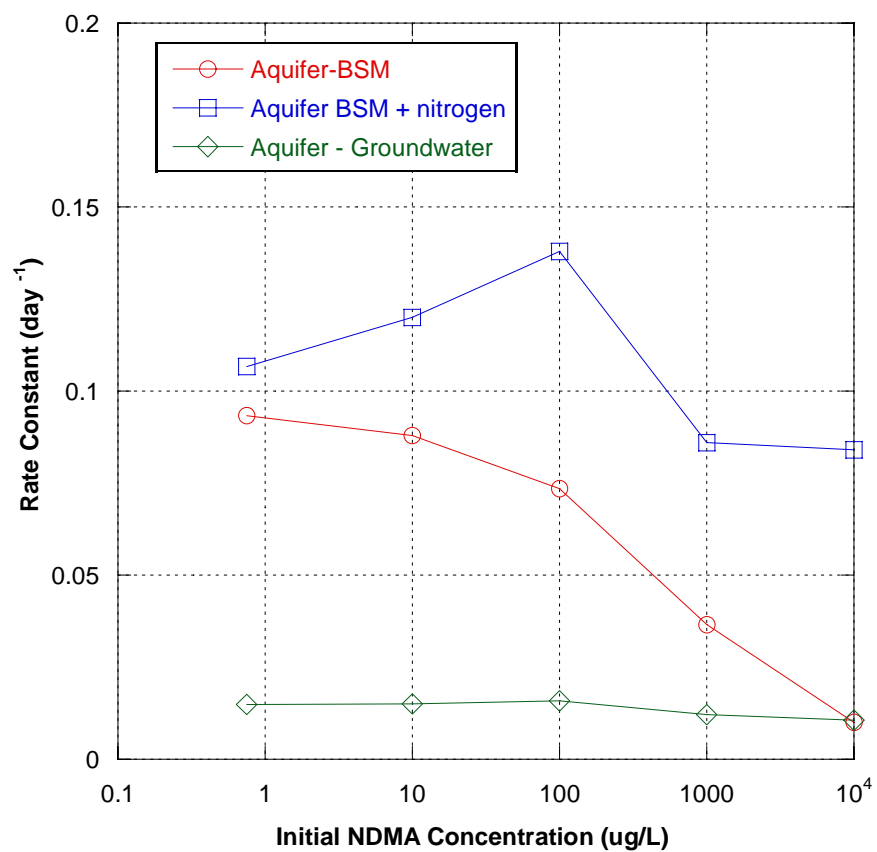


Figure 32. Comparison of mineralization rates of ^{14}C -NDMA to $^{14}\text{CO}_2$ in microcosms prepared from New Jersey aquifer sediments and BSM-K, BSM-K with nitrogen, or groundwater. Values are plotted on a log-linear scale.



4.2.2 NDMA Biodegradation under Various Electron-Accepting Conditions

Indigenous microorganisms present in a variety of different environments, including surface soil, pond sediment, sewage sludge, manure, and some aquifers were capable of mineralizing appreciable quantities of ^{14}C -NDMA to $^{14}\text{CO}_2$ (Section 4.2.1). We hypothesize that much of this activity is co-metabolism, rather than growth-linked NDMA degradation (i.e., cells grow on an alternate carbon source and degrade NDMA fortuitously through general oxidase enzymes or other non-specific metabolism). Additional studies were performed to evaluate the potential for anaerobic degradation of NDMA under different electron-accepting conditions, including nitrate-reducing, sulfate-reducing, iron-reducing, manganese-reducing, and methanogenic conditions. A series of experiments was also conducted to better understand NDMA mineralization under aerobic conditions, including the role of alternate carbon sources, nutrients, and various oxidase enzymes in supporting this activity.

4.2.2.1 Materials and Methods: Electron-Accepting Conditions

Microcosms were prepared to stimulate different electron-accepting conditions, including aerobic, denitrifying, sulfate-reducing, iron-reducing, manganese-reducing, and methanogenic conditions. In these studies, specific growth media designed to support each of the various classes of organisms (e.g., sulfate-reducers) were prepared, inoculated with aquifer sediments from one of two sites (Air Force Site PJKS or the southern California Site), then amended with lactate as a primary electron donor to promote the desired electron-accepting reaction/redox conditions in each bottle. A list of the conditions tested and the specific media used for each is as follows.

Aerobic. Aerobic microcosms were set up using unamended nitrogen-free basal salts medium (BSM-K), and were set up both with and without lactate.

Nitrate-Reducing. To support nitrate-reducing conditions, a nitrogen-free basal salts medium was amended with 0.75 g/L potassium nitrate as the electron acceptor (Eriksson et al, 2003).

Sulfate-Reducing. To induce sulfate-reducing conditions, nitrogen-free basal salts medium (BSM-K) with trace metals was amended with 100 mg/L sulfate (as sodium sulfate). At the time of inoculation, 0.24 mg/L sodium sulfide was added.

Iron-Reducing. To induce iron-reducing conditions, FWA-Fe(III) citrate medium was prepared as described by Lovley and Phillips, 1988.

Manganese-Reducing. Mineralization of NDMA under manganese-reducing conditions was tested in FWA-Mn(IV) medium, which was prepared as described in Lovley and Phillips (1988), except that MnO₂ was purchased commercially from Riedel-deHaen (Steinheim, Germany).

Methanogenic. For methanogenic conditions, a medium designed to sustain methanogenic bacteria was prepared as described by Edwards and Grbic-Galic (1994).

All anaerobic media were boiled under nitrogen gas for 20 min to remove oxygen, and then removed to a Coy anaerobic chamber with a nitrogen headspace to cool. The media were then pipetted into serum vials, which were sealed with airtight stoppers, and autoclaved. The vials then received a solution containing non-radiolabeled NDMA (to achieve a final concentration of ~ 50 ug/L) mixed with 0.05 µCi [¹⁴C]-NDMA (specific activity = 55 mCi/mmol). A small glass test tube containing 1 mL of 0.5 N sodium hydroxide was used as a base trap for ¹⁴CO₂. Samples were placed on a shaker in the dark at room temperature (28°C) and shaken at ~ 100 rpm. The various media were inoculated with 0.5 g of homogenized aquifer sediment from either the southern California site or the New Jersey. All anaerobic microcosms received 500 mg/L lactate. Controls consisted of formaldehyde-killed microcosms (final concentration of formaldehyde of 3% vol/vol). Serum bottles in which ¹⁴C-NDMA was omitted were also prepared and analyzed for anions via EPA Method 300.0 (ion chromatography).

Sample collection and analysis

For microcosm studies in which NDMA mineralization was quantified, the vials were sampled periodically (usually twice weekly) by removing the total volume of base from the base trap to a glass scintillation vial. The base trap was then refilled with fresh base. A 10 mL volume of OptiPhase Hi-Safe 3 scintillation cocktail (Perkin Elmer, Loughborough, England) was added to each vial. The vials were then mixed by vortex and the radioactivity was counted using a Rackbeta Model 1209 scintillation counter (Pharmacia LKB Nuclear, Gaithersburg, MD) programmed for analysis of ¹⁴C. Each vial was counted for 1 min.

4.2.2.2 Results and Discussion: Electron-Accepting Conditions

Southern California

As expected based on our previous results, NDMA was degraded most rapidly in aerobic microcosms (Figure 33). Interestingly, in the aerobic bottles receiving lactate, the rate and percent of NDMA mineralization was appreciably higher than in the aerobic

microcosm with no exogenous carbon source. Degradation without lactate was significantly slower, but more than 39% of the initial NDMA added was degraded over 21 days. These data further support the hypothesis that aerobic mineralization of NDMA is carried out either co-metabolically or in order to obtain nitrogen for growth (or some combination of the two mechanisms). The addition of lactate could stimulate either mechanism.

Significant NDMA mineralization was also observed under sulfate-reducing conditions in microcosms prepared from southern California site samples (Figure 34). Greater than 50% of the added NDMA was degraded in media designed to stimulate sulfate-reducing conditions after 35 days. NDMA degradation was not observed in media produced to generate iron-reducing, manganese-reducing, denitrifying, or methanogenic conditions. It should be noted that lactate was added to each of the media as a carbon source, and also to generate the appropriate electron accepting conditions.

PJKS

In samples amended with solids from the PJKS aquifer, NDMA was degraded most rapidly in aerobic microcosms (Figure 35). In the aerobic bottles receiving lactate, the rate and extent of NDMA mineralization was appreciably higher than in the aerobic microcosm with no exogenous carbon source. Degradation without lactate was significantly slower, with only ~ 14% of the initial NDMA added was degraded over 20 days, compared to over 30% mineralized in the lactate-amended treatments in the same time period. Degradation in both aerobic treatments (with and without lactate) ceased after 20 days. These data further support the hypothesis that aerobic mineralization of NDMA is carried out either co-metabolically or to obtain nitrogen for growth (or some combination of the two mechanisms). The addition of lactate could stimulate either mechanism.

Unlike our previous redox study using sediment inoculum from a southern California site (see Figure 34), no significant NDMA mineralization was observed under sulfate-reducing conditions using sediment from the Colorado site. As in the previous study, mineralization was also not observed under nitrate-reducing, manganese-reducing, iron-reducing, or methanogenic conditions. Thus, the data suggest that NDMA mineralization under sulfate-reducing conditions is not universal, but rather occurs on a site-specific basis.

Figure 33. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ under aerobic conditions in the presence or absence of lactate as a cosubstrate. Microcosms were prepared from groundwater and aquifer solids collected from the California site.

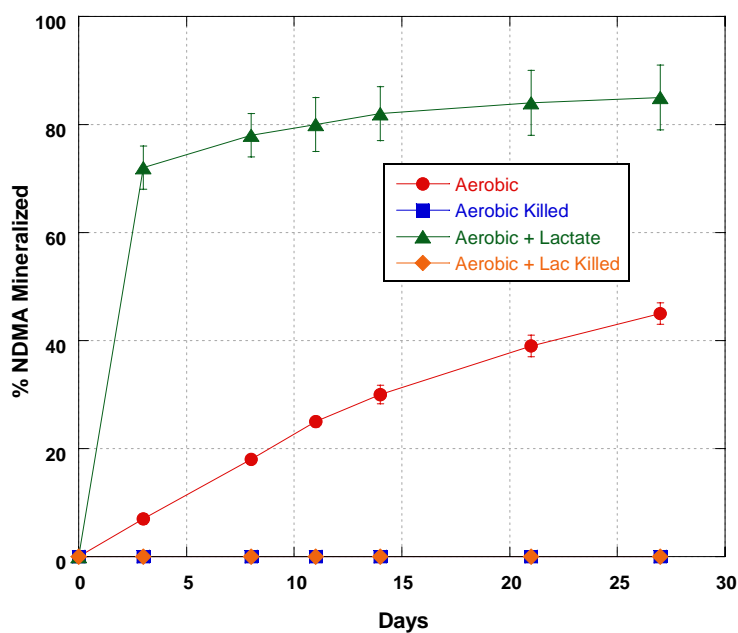


Figure 34. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ under differing terminal electron-accepting conditions: California site. Samples were inoculated with aquifer solids from a site in southern California.

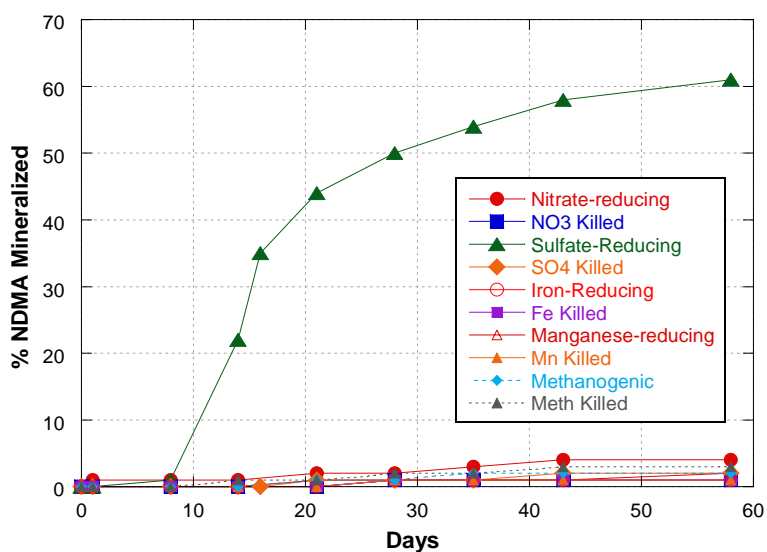
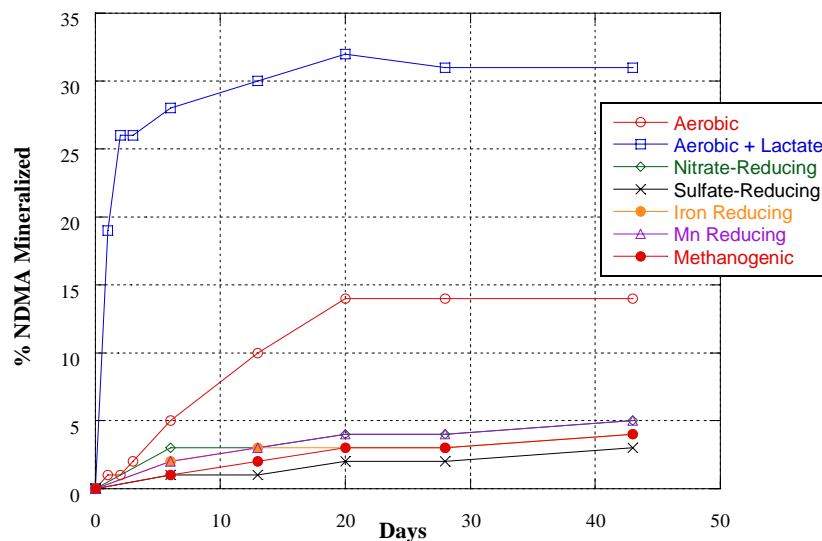


Figure 35. Mineralization of ^{14}C -NDMA to $^{14}\text{CO}_2$ under differing terminal electron-accepting conditions. Samples were inoculated with aquifer solids at the PJKS site in Colorado and amended with lactate.



4.2.3 NDMA Biodegradation in Anaerobic Groundwater

Numerous studies were conducted during this SERDP Project with aquifer samples collected from Air Force Plant PJKS (PJKS) in Colorado (see Sections 4.2.1 & 4.2.2). As part of their effort to stimulate *in situ* reductive dechlorination of TCE, contractors at PJKS injected lactate (and in some cases *Dehalococcoides* spp.) at several locations in 2006. Some of the plumes with TCE also have commingled NDMA. Quarterly sampling data revealed significant reductions in TCE levels in lactate-amended groundwater with no appreciable decline in NDMA during the initial several quarterly sampling events. However, beginning in early- to mid-2007, several wells appeared to show a slow but significant decline in NDMA from starting levels of ~1-3 $\mu\text{g/L}$ down to ~0.5 $\mu\text{g/L}$ (Figure 36). Based on this field observation, two laboratory studies were conducted to determine whether anaerobic degradation of NDMA could be verified in a controlled setting. In our earlier anaerobic studies using materials from this site (see Figure 35), no NDMA mineralization was observed under anaerobic conditions. These earlier studies, however, only measured complete mineralization of NDMA to carbon dioxide, and thus did not account for potential NDMA biodegradation to other intermediates (e.g., methane).

4.2.3.1 Materials and Methods: NDMA Biodegradation in Anaerobic Groundwater

In the initial study, groundwater collected from well BCMW-019-P at PJKS (see Figure 36 for historical well data) was added in 25-mL aliquots to sterile 160-mL serum bottles. All work was conducted in a Coy Environmental Chamber with a N₂ headspace to exclude oxygen. Nine bottles received 10 µg/L of NDMA and 50,000 dpm of ¹⁴C-NDMA. Triplicate bottles then received either lactate (to 50 mg/L final concentration), no addition, or formaldehyde as a killing agent (2% vol/vol). A Durham tube containing 1 mL of 0.5 N NaOH was then added to each bottle to trap ¹⁴CO₂ evolved from mineralization of the ¹⁴C-NDMA. Bottles were incubated anaerobically at room temperature in the dark with gentle shaking.

In a second study, large samples were prepared to measure actual loss of NDMA, rather than production of ¹⁴CO₂. Three 4-L bottles containing 3L PJKS site groundwater were used for this study. Prior to study setup, the bottles were cleaned, equipped with Teflon-lined caps, and autoclaved. After autoclaving, the sterile bottles were moved into a Coy Environmental Chamber, where the remainder of the experimental setup was performed in a N₂ headspace. All bottles received 3L of site groundwater. The bottles were then incubated for 24 hrs in the chamber to ensure anaerobic conditions. After 24 hrs, three treatments were prepared: (1) Live Control, which received 10 µg/L NDMA only; (2) Killed Control, which received 10 µg/L NDMA plus 2% formaldehyde (v/v); and (3) Biostimulation, which received 10 µg/L NDMA plus 100 mg/L lactate. Bottles were incubated at room temperature. Samples were analyzed for NDMA in Shaw's analytical laboratory by GC-MS to evaluate initial concentrations. After 42 days, a 500 mL sample was removed from each bottle and sent to Maxxam Analytics for low-level NDMA analysis via HRMS/MS (see Section 4.4.2.2 for method details).

4.2.3.2 Results and Discussion: NDMA Biodegradation in Anaerobic Groundwater

During 42 days of incubation, less than 5% of the NDMA added to site groundwater was converted to CO₂ in live samples or those amended with additional lactate (Figure 37). There was no difference in NDMA conversion between the live samples and formaldehyde-killed controls. There was also no indication of biological NDMA degradation in the large-scale microcosms. However, > 50% loss of NDMA was observed in all bottles from Day 0 to Day 42, including the formaldehyde-treated samples (Figure 38). The initial concentration of NDMA in the groundwater, which averaged ~ 14 µg/L, ranged from 4.8 to 6.3 µg/L after 42 d of incubation. It is possible that the decline in NDMA concentration in these samples is the result of abiotic reduction reactions with dissolved iron (Fe (II)), sulfide (S²⁻), or particulate minerals containing one or both of these species. In that these reactions would likely result in reduced rather than

oxidized end-products, it is unlikely that CO₂ would be produced. Thus, this hypothesis is consistent with the results from the small-scale microcosm study presented in Figure 37.

Data from SERDP Project ER-1421 *Abiotic and Biotic Mechanisms Controlling In Situ Remediation of NDMA* (Jim Szecsody; Pacific Northwest National Laboratory; PNNL) show that abiotic reduction of NDMA in aquifer sediments is feasible. In fact, NDMA half-lives of ~ 8 hr were observed in reduced aquifer sediments collected from the Aerojet site in Sacramento, CA after treatment with dithionite as a reducing agent. However, the mineral/chemical species mediating these reactions are unclear, and may be very complex, and reduction reactions are highly sensitive to pH, being rapid at alkaline pH, and ceasing at neutral pH. One key reactive species appears to be ferrous iron adsorbed to magnetite. However, under many experimental conditions, including most studies conducted at neutral pH, abiotic reduction of NDMA did not occur. Additional studies are ongoing at PNNL to better define the chemical reactions responsible for abiotic reduction of NDMA. Thus, it is feasible that the reduction of NDMA in the PJKS samples is the result of one or more abiotic reactions. However, additional work in this area was beyond the scope of this project, which focused largely on biotic reactions.

Figure 36. Concentrations of NDMA in groundwater collected from PJKS during the past 3 years. The area was treated with sodium lactate to create reducing conditions and stimulate reductive dechlorination in 2006.

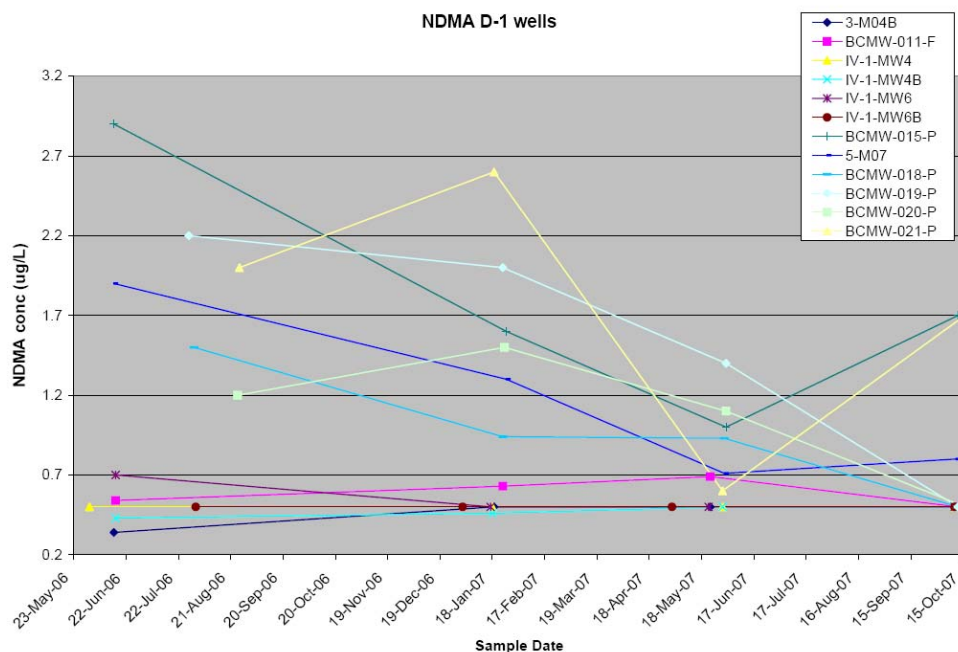


Figure 37. Mineralization of NDMA in groundwater samples from Well BCMW-019-P at PJKS. Error bars are smaller than symbols.

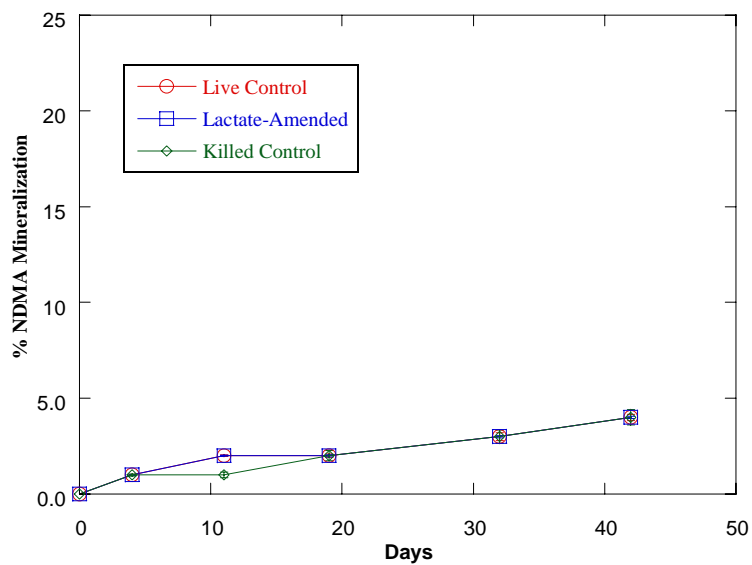
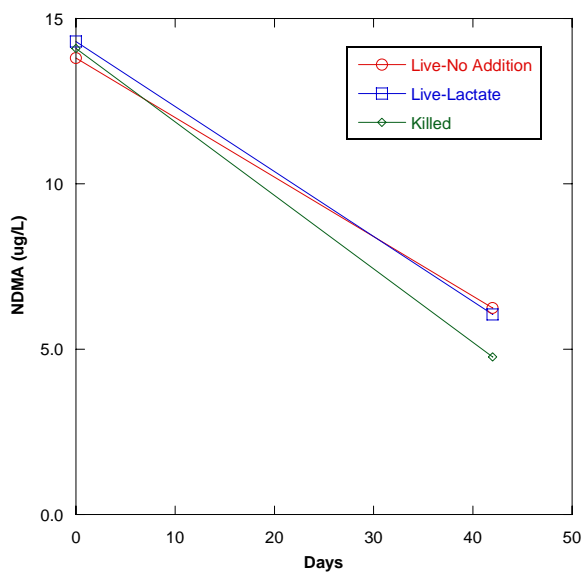


Figure 38. Concentrations of NDMA in large groundwater microcosms with no addition, lactate, or formaldehyde (killed control).



4.3 Culture Enrichment and Isolation

Individual bacterial colonies were isolated from samples showing NDMA mineralization under aerobic conditions. NDMA was added as a sole carbon and/or nitrogen source. The ability of these individual isolates to metabolize NDMA in the presence and absence of exogenous carbon was then tested. In a second set of studies, aquifer samples from all three sites (southern California, PJKS, and New Jersey) were incubated with propane for three weeks in the absence of NDMA. NDMA was then added to these samples and extensive mineralization was observed (compared to controls receiving no propane) (see Section 4.6 for details). Enrichment cultures on propane (i.e., indigenous propanotrophs) were obtained from each of these samples, and tested for their ability to mineralize NDMA.

4.3.1 Enrichment and Isolation of Cultures Capable of Growth on NDMA

4.3.1.1 Materials and Methods: Enrichment and Isolation of Cultures Capable of Growth on NDMA

Liquid subsamples (25 μ L) were removed from select aerobic microcosms in which rapid NDMA mineralization was observed. These microcosms included bottles that were prepared from a NJ farm soil and from various aquifer samples (New Jersey, Air Force Plant PJKS, southern California site) as detailed in Section 4.2. Some samples were added to sterile serum bottles containing BSM-K with 10 mg/L NDMA. These bottles were placed in the dark on a shaker at room temperature and checked periodically for turbidity as an indication of bacterial growth.

Other liquid subsamples were streaked onto solid R2A media (Becton, Dickinson and Company, Sparks, MD), and the agar plates were incubated at 22°C until colonies were observed. Individual colonies were selected using a stereomicroscope and streaked onto fresh plates of R2A agar. This procedure was repeated until a pure culture was obtained based on colony morphology. For mineralization studies, bacterial colonies from each plate were inoculated into liquid Tryptic Soy Broth (TSB; Becton, Dickinson and Company) and grown overnight on a rotary shaker. The cultures were centrifuged, resuspended in BSM, and then inoculated into 160-ml serum bottles containing either 50 μ g/L or 5 mg/L non-radiolabeled NDMA and 0.02 μ Ci 14 C-NDMA. Three different conditions were tested:

- (1) Complete basal salts medium (BSM-H); Cells were resuspended in basal salts containing all necessary inorganic macro and micronutrients with no exogenous carbon source. The ability of cells to use NDMA as a carbon source (and mineralize the nitrosamine) was tested in this treatment.

(2) Nitrogen free plus carbon source (BSM-K + lactate): Cells were suspended in nitrogen-free basal salts medium containing 500 mg/L lactate (as sodium lactate) as a carbon source. This condition was used to determine if cells could use NDMA as a nitrogen source while growing on lactate

(3) Nitrogen-free media (BSM-K): Cells were resuspended in nitrogen-free basal salts media with no exogenous carbon source. The ability of cells to use NDMA as a carbon and nitrogen source was tested in this treatment.

A test tube containing 0.05 NaOH was added to each vial to trap any $^{14}\text{CO}_2$ released during NDMA mineralization. Bottles were incubated for 5 days with gentle shaking, after which time the NaOH in each base trap was removed and measured for radioactivity in a liquid scintillation counter. The presence of radioactivity above the background measured in killed control samples indicates $^{14}\text{CO}_2$ evolution from ^{14}C -NDMA.

4.3.1.2 Results and Discussion: Enrichment and Isolation of Cultures Capable of Growth on NDMA

None of the enrichments receiving 10 mg/L NDMA and subsamples from various locations showed increased turbidity after more than 3 months of incubation. Some bottles were treated a second time with 10 mg/L NDMA without effect.

Over 60 individual cultures were isolated and purified from the various aerobic microcosms. The ability of 19 of these isolates to mineralize NDMA was tested (10 from farm soil, 6 from New Jersey aquifer solids, and 3 from Air Force Plant PJKS aquifer solids). None of the isolates examined were observed to mineralize appreciable quantities of ^{14}C -NDMA to carbon dioxide (< 1% NDMA mineralized), irrespective of NDMA concentration or the presence or absence of exogenous nitrogen or carbon (*data not shown*).

The variety of samples in which this NDMA mineralization was observed under aerobic conditions and the high extents of mineralization (see previous quarterly reports) indicate that bacterial cultures capable of performing this activity exist, and are potentially widespread. However, none of the environmental isolates we that we purified were capable of utilizing NDMA as a sole C and N source. This result may reflect the enrichment and selection conditions (e.g., transfer from NDMA-amended microcosms to R2A agar) or the fact that microbial consortia rather than individual cells are responsible for NDMA mineralization in many environments. It is possible that some of our isolates were capable of partially oxidizing NDMA, but not oxidizing the carbon all the way to carbon dioxide. We did not measure loss of NDMA in these studies. The fact that no other environmental isolates capable of growing on NDMA (i.e., utilizing NDMA as a sole source of carbon and energy) have been reported in the literature suggests that NDMA mineralization in nature may require the cooperative activity of multiple

organisms, reflect co-metabolic activity of one or more strains, or may otherwise be a complex process. Additional studies are necessary to evaluate these possibilities.

4.3.2 Enrichment and Isolation of Propanotrophs from Aquifer Samples

4.3.2.1 Materials and Methods: Propanotrophs

Previous experiments in our laboratory demonstrated that several propanotrophs, including *R. ruber* ENV425 (see section 4.1.4) and *M. vaccae* JOB5, are capable of mineralizing NDMA. The purpose of this phase of work was to determine whether indigenous propanotrophs could be enriched from various aquifer solids, and whether NDMA is mineralized by such enrichments. A second set of studies was conducted simultaneously to determine whether addition of propane (and other substrates) to aquifer materials can be used to stimulate NDMA mineralization by indigenous bacteria (see Section 4.6).

For enrichment studies, five grams of aquifer solids from New Jersey, PJKS, or the southern California site were placed into 250 mL flasks containing 100 mL of basal salts medium (BSM-H). Each flask was then connected to a Tedlar bag filled with a gas mixture consisting of 25% propane and 75% oxygen. Flasks were shaken at ambient temperature, and were tested periodically for propane utilization. After ~ 7.5 weeks of incubation, 10 mL of liquid from each flask was transferred to a fresh sterile 250-mL bottle with 200 mL BSM-H and a propane/oxygen headspace. To test for NDMA mineralization, a 25 mL volume of each enrichment culture was added to a sterile 160-mL serum bottle containing a base trap (1 mL of 0.5N NaOH) to collect $^{14}\text{CO}_2$. Each bottle was then amended with 50 $\mu\text{g/L}$ of cold NDMA and approximately 50,000 dpm of ^{14}C -NDMA. The headspace was then filled with 60 mL of a gas mixture containing 25% propane and 75% oxygen. One bottle of each enrichment was treated with 670 μL of 37% formaldehyde solution as a killing agent (i.e., killed control). All bottles were placed at room temperature and shaken at 100 rpm. The NaOH in each trap was sampled periodically to assess mineralization of ^{14}C -NDMA. Dilutions of each enrichment were subsequently transferred to solid agar plates (BSM-H). These plates were then placed in “anaerobic” incubation jars (gas-tight), which received a headspace of 25% propane and 75% oxygen. The pure cultures ENV-PIC1, ENV-PIC3 (both isolated from New Jersey site materials), and ENV-SC1 (isolated from southern California site materials) were obtained this way.

The pure cultures ENV-PIC1, ENV-PIC3, and ENV-SC1 were grown in BSM-H with ~10% propane (v/v) in the headspace. The headspace of these cultures was flushed with air and the propane was replaced weekly. After 4 weeks, each of the cultures showed significant growth (OD_{600} = 0.425 for ENV-PIC1, 0.233 for ENV-PIC3 and 0.86 for ENV-SC1) on propane as a sole carbon source. Thirty mL of each culture was then transferred to two sterile 160-mL serum bottles, one of which was treated with 2%

formaldehyde as a killing agent. Each bottle then received 2 mg/L of NDMA. After 0, 1, 2, and 6 days of incubation, 5 mL of BSM was removed from each bottle to a clean glass vial, and each sample was frozen. The samples were then extracted with methylene chloride and analyzed for NDMA via GC/MS.

4.3.2.2 Results and Discussion: Propanotrophs

After 1 week of incubation, the flask inoculated with solids from the PJKS site exhibited signs of propane utilization (measured by loss of gas from the Tedlar bag), and by 3 weeks the flask containing New Jersey solids also exhibited signs of propane loss. By 7.5 weeks (when the samples were serially transferred), propane utilization was also observed in the flask seeded with southern California sediments. Two weeks after transfer to fresh media, microbial growth was observed in all three enrichments. The ability of each propane enrichment culture to mineralize NDMA was then tested. After 3 days of incubation, the enrichment cultures from each site mineralized > 75% of the 50 µg/L of NDMA added to each serum bottle (*data not shown*). This mineralization occurred in the presence of propane, which was added to the headspace of each bottle. In contrast, no NDMA mineralization was observed in the killed controls. Dilutions of each enrichment were subsequently transferred to solid agar plates (BSM-H). These plates were then placed in “anaerobic” incubation jars (gas-tight), which received a headspace of 25% propane and 75% oxygen. The pure cultures ENV-PIC1, ENV-PIC3 (both isolated from New Jersey site materials), and ENV-SC1 (isolated from southern California site materials) were obtained this way. (A fourth pure culture, denoted ENV-PJKS1 was later isolated from Air Force Plant PJKS materials, but the strain was extremely slow-growing and work with it was discontinued).

Analysis of NDMA in sample bottles by GC/MS confirmed that all three propanotrophs are capable of rapidly biodegrading NDMA. The three strains were identified using 16S rDNA sequencing. All three strains were identified as *Nocardioides spp.* (Figure 39a-c). Interestingly, ENV-PIC1, which was isolated from the New Jersey aquifer samples, and ENV-SC1, which was isolated from the site in southern California, had nearly identical 16S rDNA profiles, while ENV-PIC3 was slightly different. Other *Nocardioides* species are capable of degrading compounds such as 2,4,6-trinitrophenol (picric acid) and 2,4-dinitrophenol (Ebert et al, 1999), phenanthrene (Saito et al, 2000), *S*-triazine (Topp et al, 2000), and numerous alkanes (Hamamura et al, 2001). Many of these strains were shown to contain oxygenase enzymes (Saito et al, 2000; Hamamura et al, 2001), so the ability of a *Nocardioides spp.* to degrade NDMA is not particularly surprising. It is interesting however, that all three strains of propanotrophs isolated during the course of this project were of this genus. It appears that NDMA co-metabolism is a common trait among propanotrophic bacteria, presumably due to the similarity in propane monooxygenase genes/enzymes among these various organisms.

The enrichment data suggest that a widely distributed group of propanotrophs (or a ubiquitous species) is capable of mineralizing NDMA. When incubated with propane for several weeks, bacteria indigenous to aquifer samples from Colorado, New Jersey, and California were observed to rapidly biodegrade small quantities of NDMA. NDMA mineralization in untreated samples in these studies was low by comparison (*data not shown*). Moreover, the presence of propane (the primary growth substrate) does not appear to significantly inhibit NDMA biodegradation in these studies – in fact, stimulation is generally observed. Competitive inhibition has previously caused many co-metabolic processes to be difficult to implement in the field for bioremediation purposes (i.e., because the parent growth substrate inhibits biodegradation of the contaminant-of-concern). Biostimulation with propane may be an excellent alternative to treat NDMA in some groundwater aquifers as well as in *ex situ* bioreactors (see Sections 4.4 & 4.6).

Figure 39a. Identification of bacterial isolate PIC1 based on 16S rDNA analysis. Results are based on sequence analysis of 500 base pairs.

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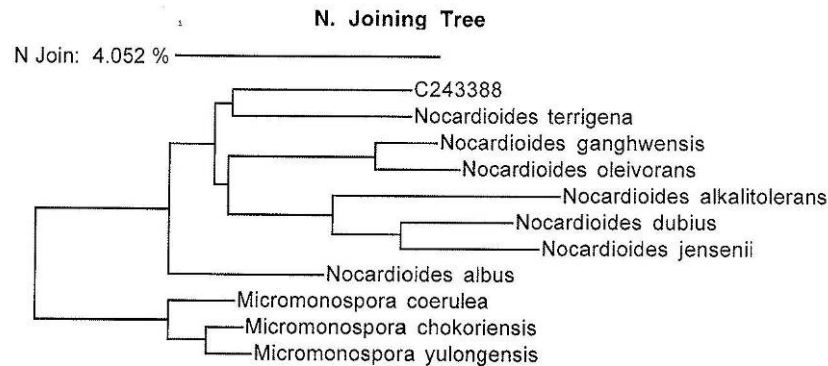
IDENTIFICATION REPORT
SOP-GEN-017.1

Company: Shaw Environmental, Inc.

Sample: C243388 - PIC 1

Date: Thursday, August 2, 2007 3:24 PM

Alignment: 503 C243388
5.39 % 501 Nocardioiodes terrigena
5.60 % 500 Nocardioiodes albus
6.37 % 502 Nocardioiodes ganghwensis
6.79 % 501 Nocardioiodes oleivorans
7.36 % 503 Nocardioiodes dubius
7.95 % 503 Nocardioiodes jensenii
8.18 % 501 Nocardioiodes alkalitolerans
9.02 % 499 Micromonospora chokoriensis
9.42 % 499 Micromonospora coerulea
9.42 % 499 Micromonospora yulongensis



Accugenix Database Search Result

Generated by: ap2Aug07 Identification: Nocardioiodes sp.

Reviewed by: SM02Aug07 Confidence Level: Genus

QA Review: M402Aug07

Batch #: 70802021

EVG1 - Due 8/9/07

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Figure 39b. Identification of bacterial isolate PIC3 based on 16S rDNA analysis. Results are based on sequence analysis of 500 base pairs.

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Company: Shaw Environmental, Inc.

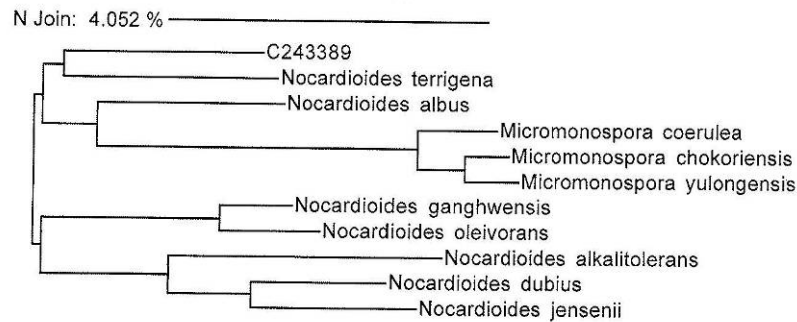
Sample: C243389 - PIC 3

Date: Thursday, August 2, 2007 3:24 PM

Alignment: 502 C243389

5.19 % 501 Nocardioiodes terrigena
5.40 % 500 Nocardioiodes albus
6.18 % 502 Nocardioiodes ganghwensis
6.59 % 501 Nocardioiodes oleivorans
7.17 % 503 Nocardioiodes dubius
7.77 % 503 Nocardioiodes jensenii
7.98 % 501 Nocardioiodes alkalitolerans
8.82 % 499 Micromonospora chokoriensis
9.22 % 499 Micromonospora yulongensis
9.22 % 499 Micromonospora coerulea

N. Joining Tree



Accugenix Database Search Result

Generated by: cpo2 Aug 02

Identification: Nocardioiodes sp.

Reviewed by: DMU2 Aug 07

Confidence Level: Genus

QA Review: Muto Aug 07

Batch #: 70802021

EVG1 - Due 8/9/07

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Rev. 01Nov2006 JDB

Figure 39c. Identification of bacterial isolate SC1 based on 16S rDNA analysis. Results are based on sequence analysis of 500 base pairs.

ACCUGENIX

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Newark, DE 19702
PHONE (302) 282-8888
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IDENTIFICATION REPORT
SOP-GEN-017.1

Company: Shaw Environmental, Inc.

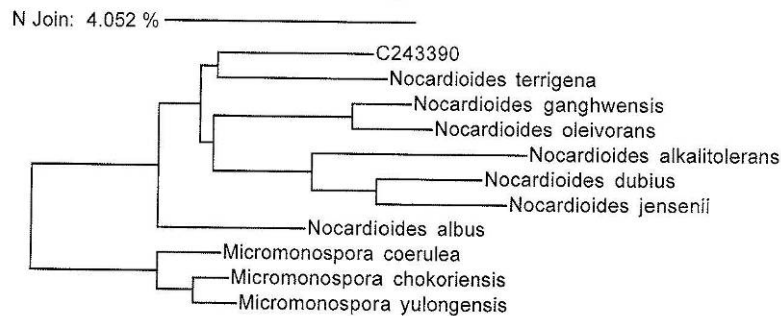
Sample: C243390 - SC1

Date: Thursday, August 2, 2007 3:25 PM

Alignment: 502 C243390

5.19 % 501 Nocardioides terrigena
5.40 % 500 Nocardioides albus
6.18 % 502 Nocardioides ganghwensis
6.59 % 501 Nocardioides oleivorans
7.17 % 503 Nocardioides dubius
7.77 % 503 Nocardioides jensenii
7.98 % 501 Nocardioides alkalitolerans
8.82 % 499 Micromonospora chokoriensis
9.22 % 499 Micromonospora yulongensis
9.22 % 499 Micromonospora coerulea

N. Joining Tree



Accugenix Database Search Result

Generated by: *[Signature]*

Identification: Nocardioides sp.

Reviewed by: *[Signature]*

Confidence Level: Genus

QA Review: *[Signature]*

Batch #: 70802021

EVG1 - Due 8/9/07

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4.3.3 Evaluation of NDMA Biodegradation by Cultures Isolated for Degradation of N,N-dimethylformamide (DMF) and N-methyl-2-pyrrolidone (NMP).

During the course of this project, we have isolated three new strains and identified several existing strains capable of cometabolizing NDMA after growth on one or more primary substrates (e.g., propane, methane, toluene, etc). No strain isolated in our laboratory or any others has yet been shown to grow (i.e., obtain carbon and energy) during NDMA biodegradation. Recently, we developed microbial consortia capable of metabolizing N,N-dimethylformamide (DMF), (Shaw culture ENV-DMF), and N-methyl-2-pyrrolidone (NMP), (Shaw culture ENV-NMP). Because there are similarities in the chemical structures of DMF and NMP to NDMA (particularly DMF as shown in Figure 40), we evaluated NDMA mineralization by each of these mixed cultures.

4.3.3.1 Materials and Methods: NDMA Degradation by Cultures ENV-DMF and ENV-NMP

Cultures were initially grown aerobically to an OD₅₅₀ greater than 100. This was conducted in a commercial fermentor. Each culture was diluted in BSM-H without nitrogen (no the ammonium added). The nitrogen was removed from the BSM because the primary cultures were thought to use DMF and NMP as a nitrogen as well as a carbon source. Ten mL of each culture was placed into three sterile 160-mL serum bottles. The following treatments were prepared: (1) live cells; (2) live cells with 1 mg/L of either DMF or NMP as a cosubstrate; (3) killed cells (treated with 1% formaldehyde (v/v)). All bottles received 500 µg/L unlabeled NDMA and ~ 50,000 dpm of ¹⁴C-NDMA. A Durham tube containing 1 mL of 0.5 N NaOH was then added to each bottle to trap ¹⁴CO₂ evolved from mineralization of the ¹⁴C-NDMA. Bottles were incubated aerobically at room temperature in the dark with gentle shaking.

4.3.3.2 Results and Discussion: NDMA Degradation by Cultures ENV-DMF and ENV-NMP

Both cultures, ENV-DMF and ENV-NMP, mineralized ¹⁴C-NDMA to ¹⁴CO₂ (Figure 41). Moreover, the presence or absence of cosubstrate made little difference in the quantities of NDMA mineralized or the rate of mineralization. Interestingly, however, the NDMA mineralization curves were very different between the two cultures. ENV-NMP degraded NDMA immediately with no apparent lag period; 30% of the added NDMA mineralized in the first 2 days of incubation, and after that time, the NDMA degradation rate declined significantly. In contrast, culture ENV-DMF demonstrated a lag period of 2 to 5 days, after which NDMA mineralization proceeded at a rapid rate until 8 days of incubation. The shape and trends seen in ENV-DMF's mineralization curve are indicative of a growth curve and indicate that ENV-DMF may be capable of utilizing NDMA as a source of carbon, energy, and possibly nitrogen. Confirmation is required using NDMA

as a sole growth substrate in the presence or absence of nitrogen. However, it appears that both consortia are capable of biodegrading NDMA.

Figure 40. Chemical structures of NDMA, DMF, and NMP.

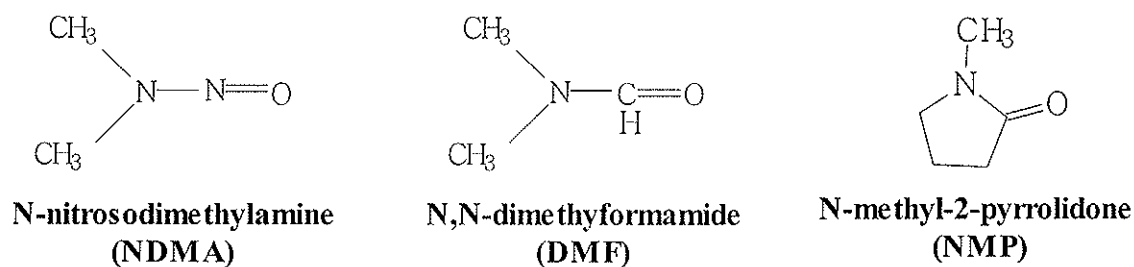
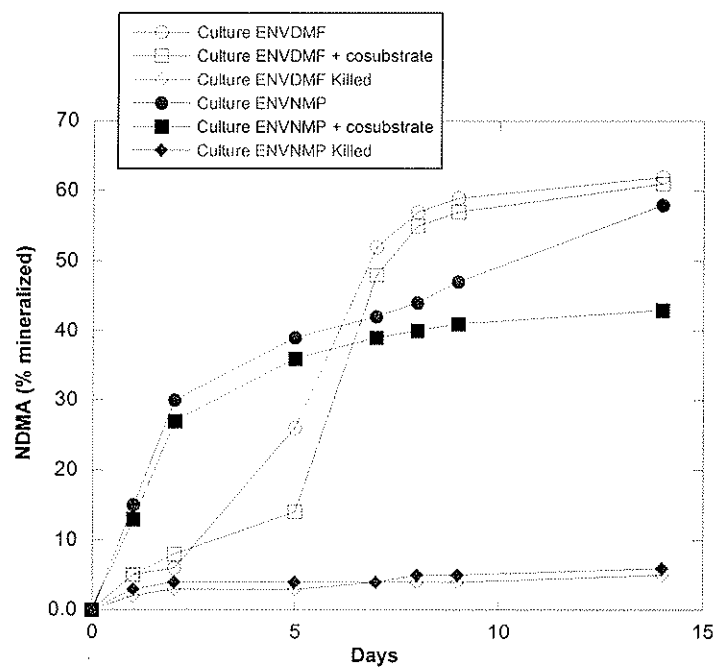


Figure 41. NDMA mineralization by culture ENV-DMF and ENV-NMP in the presence or absence of cosubstrates.



4.3.4 Evaluation of NDMA Degradation by Xen A and Xen B Enzymes

The potential for the enzymes Xenobiotic Reductase A (XenA) and Xenobiotic Reductase B (XenB) to catalyze the degradation of NDMA was investigated. These enzymes, which have a broad substrate specificity, have previously been found to degrade nitramines and nitroaromatics such as RDX and TNT, respectively. No tests have been performed to evaluate the activity of these enzymes on nitrosamines.

4.3.6.1 Methods: Xen A and Xen B Enzymes

Experiments were performed to assess the catalytic properties of the xenobiotic reductases of *P. putida* II-B and *P. fluorescens* I-C, which were purified essentially as previously described (Bleher et al., 1997; Pak et al., 2000). NDMA degradation assays were performed with NDMA dissolved in sodium phosphate buffer (final concentration 50 mg/L, pH 7.4). Reductant in the form of NADPH⁺ was added to a final concentration of 1 to 2 mg/mL. Vials were purged with at least twenty volumes of oxygen-free nitrogen bubbled through the liquid, then transferred to an anaerobic chamber where 1 mL of the solutions were transferred to 2 mL glass screw cap auto-sampler vials and sealed with Teflon lined septa. The assays were initiated by injecting 1 µL of purified XenB (0.017 mg) or 5 µL of purified XenA (0.070 mg) through the septa. End-point experiments were incubated for no less than 24 hrs prior to analysis. Negative controls comprised of substrate, buffer, and NADPH⁺ were included in all experiments, and were used to detect and adjust for any non-enzymatic substrate losses. NDMA was analyzed by solvent extraction (1:1 vol:vol: with methylene chloride) and analyzed by GC/MS as described in previous reports.

4.3.6.2 Results and Discussion: Xen A and Xen B Enzymes

NDMA degradation was not observed by either of the two catalytic enzymes tested, XenA or XenB, leading us to conclude that neither of these enzymes utilizes NDMA as a substrate (*data not shown*).

4.4 Evaluation of NDMA Treatment in a Laboratory-Scale Membrane Bioreactor (MBR)

4.4.1 Batch Evaluation of NDMA Degradation by *R. ruber* ENV425

4.4.1.1 Materials and Methods: Batch Evaluation of NDMA Degradation by *R. ruber* ENV425

For a bioreactor to be applicable for NDMA treatment, influent NDMA concentrations of a few to a few hundred µg/L must be reduced to low ng/L concentrations in the effluent. Prior to commencing a bioreactor study, an initial batch study was performed to determine whether the propanotroph *R. ruber* ENV425 was capable of degrading NDMA to ng/L levels from a starting concentration of ~ 10 µg/L. A freshly-grown culture was

initially added to a 5 L volume of BSM-H attached to a 12 L Tedlar bag with 25% propane and 75% oxygen. After 12 days, when the culture density reached 1.0 (OD₅₅₀), the cell suspension was amended with NDMA to achieve a final concentration of ~ 10 µg/L. After 18, 72, and 166 hr, a 1L-volume of the culture was filtered through a Corning disposable filter unit to remove cells, then the samples were sent to Maxxam Analytics, Inc. for analysis of trace level NDMA by GC/MS/MS (see Section 4.4.2.2). Preliminary studies were conducted to ensure that NDMA was not absorbed by the filter membrane or lost during storage in jars prior to analysis. In these studies, NDMA was quantitatively recovered after membrane filtration and no loss was observed more than 1 month of storage in the filter jar in the dark (*data not shown*).

4.4.1.2 Results and Discussion: Batch Evaluation of NDMA Degradation by *R. ruber* ENV425

The initial concentration of NDMA added to the 5L-volume of cells was ~ 8.3 µg/L. This value was obtained by spiking a liter of sterile water with NDMA at the same time as the culture flask. At 18 hrs and at each sample point thereafter, the NDMA concentration in solution was determined to be < 2 ng/L (the PQL for the analysis) (Figure 42). These data show that under batch conditions, strain ENV425 is capable of degrading NDMA to below current detection limits of NDMA analysis (i.e., 2 ng/L), and they provide a basis to move forward with bioreactor studies with this organism. The ability of this or any other bacterium to achieve low ng/L concentrations of NDMA is important if bioremediation is to be considered as a potential remedial option for NDMA in the future. The current action level for NDMA in California is 10 ng/L and the California Office of Environmental Health Hazard Assessment (OEEHA) recently recommended 3 ng/L as a public health goal (PHG) based on risk assessment (OEEHA, 2006). Bioremediation is not generally considered to achieve the reduction of contaminants from µg/L to ng/L concentrations in groundwater. However, this study reveals that, with propane as a primary growth substrate, ENV425 is capable of reducing NDMA to low ng/L concentrations. Thus, the data suggest that *in situ* and *ex situ* bioremediation with ENV425 and possibly other propanotrophs may have application for NDMA treatment.

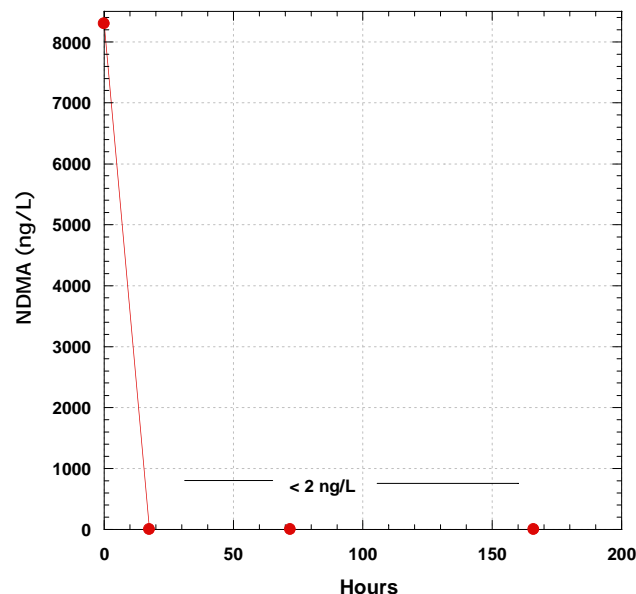
4.4.2 Propane-Fed Membrane Bioreactor (MBR)

Strain ENV425 was studied extensively during the first several months of this project, and was subsequently chosen for the reactor study. As previously noted, the strain completely mineralizes NDMA and can achieve < 2 ng/L levels in batch. Also, and perhaps most importantly, propane does not appear to be a significant competitive inhibitor of NDMA degradation in this strain despite the fact that this is a co-metabolic reaction presumed to be mediated by a propane monooxygenase (PMO). In fact, based on recent data in our laboratory, the presence of propane actually stimulates the rate of

degradation of NDMA and decreases the lag period prior to the onset of the reaction (see Figure 13). This is a critical difference from many other co-metabolic reactions in which the growth substrate inhibits degradation of the target contaminant. Competitive inhibition has limited application of such co-metabolic processes for both *in situ* and *ex situ* remediation.

Two laboratory bioreactor designs were initially considered for this study, a GAC-based fluidized bed bioreactor (FBR) and a membrane bioreactor (MBR). The MBR was selected for this initial phase of work due to concerns with NDMA mass balance using a GAC based system. The effectiveness of any biological system is dependent on maintaining a high concentration of active biomass within the reactor. This is particularly true when these are stringent effluent requirements, such as the low ng/L concentrations required for NDMA. In the MBR, a membrane is used to maintain high biomass within a reactor, while allowing liquids to pass from the reactor as treated effluent. The MBR utilizes a cross-flow membrane filtration for the solid-liquid separation step. This provides a “positive” barrier to keep bacterial solids in the reactor and also renders a clarified effluent stream for discharge. Effluent without significant biomass is important for initial laboratory testing so that low effluent PQLs for NDMA can be achieved.

Figure 42. Biodegradation of NDMA by *R. ruber* ENV425 in batch culture.



4.4.2.1 Materials and Methods: Propane-Fed MBR

A laboratory MBR was designed and constructed at the Shaw Laboratory in Lawrenceville, NJ. A schematic and photograph of the MBR system are provided in Figures 43 & 44, respectively. The reactor consisted of a 3 L glass vessel fitted with a Zenon ZeeWeed 1 (ZW1) hollow-fiber ultra filtration membrane cartridge (General Electric Water and Process Technologies, USA) (Figure 45). A peristaltic pump was connected to the ZW1 cartridge to pull effluent water through the membrane. Because of the small membrane pore size ($\sim 0.04 \mu\text{M}$), biomass remained within the reactor vessel. The reactor was continually stirred and fed pure oxygen directly through a sparge stone. The reactor volume was controlled by a level sensor. The influent to the reactor consisted initially of an artificial groundwater spiked with $20 \mu\text{g/L}$ of NDMA. The artificial groundwater, which was based on groundwater chemistry at a site in Maryland (Schaefer et al., 2007) contained NaSO_4 (180 mg/L), NaCl (113 mg/L), NaNO_3 (60 mg/L), NaHCO_3 (40 mg/L), and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1 mg/L). The pH of the groundwater was ~ 8.3 , and additional pH control in the pilot reactor was unnecessary. The groundwater was mixed 1:1 with propane-saturated water produced in a separate vessel (Figure 43 & 44; gassing vessel). This vessel contained silicone tubing which was pressurized to ~ 15 psi with high-grade propane. The propane diffused through the tubing to the artificial groundwater to achieve a concentration of ~ 60 - 80 mg/L. After the propane-laden water and groundwater were mixed, the initial influent concentrations to the reactor were $\sim 10 \mu\text{g/L}$ for NDMA and ~ 30 mg/L for propane. The influent stream received a dilute solution of inorganic nutrients (1:20 BSM-H) prior to entering the reactor. For the initial operating period, the reactor was set to have a hydraulic residence time (HRT) of approximately 30 hrs and an infinite solids retention time (SRT). These parameters were selected to maximize the extent of NDMA degradation in the unit rather than to mimic commercial operation.

The reactor was initially operated for approximately 35 days without addition of propane or biomass to establish baseline influent and effluent NDMA concentrations. The reactor was then seeded with a liquid culture of the propanotroph *Rhodococcus ruber* ENV425 to achieve an initial optical density of ~ 1.0 (OD_{550}). During operation of the laboratory MBR, temperature, reactor pH, and reactor dissolved oxygen (DO) were generally measured 4 days per week. Temperature was measured using a glass thermometer, DO was measured with an YSI 550 DO meter (YSI Incorporated, Yellow Springs, OH), and pH was measured with an Orion 720A pH meter fit with an Accumet pH probe (Cole Parmer, Vernon Hills, IL). The propane line pressure (PSI) and the reactor headspace propane were also generally measured 4 times per week throughout the duration of the study. The influent and effluent dissolved propane concentrations were measured twice per week during the initial 6 months of reactor operation, and the influent concentrations were again measured during the final 2 months of operation. Propane concentrations in solution could not be taken during the middle phase of operation due to

mechanical failure of the GC used for this purpose. Propane concentrations in the influent and effluent were analyzed with a method that utilizes Henry's Law to determine liquid concentration from analysis of headspace gas (modified from Kambell and Vandegrift, 1998). Liquid samples (30 mL) taken from the reactor influent or effluent were diluted 1:1000 in distilled deionized water in VOA vials containing 25% head space. The dissolved gasses were allowed to equilibrate in the head space, and the samples were analyzed on a Varian 3900 GC equipped with an FID detector and a Restek RTX Alumina column (50 m, 0.53 mm ID). A 4-point standard curve derived from direct injection of varying propane concentrations was used to determine the propane in each sample.

NDMA sampling required the collection of ~ 1 L of water, which took ~ 10 hr based on the reactor flow rate. Reactor effluent samples were initially collected once per week into a specially-cleaned amber glass bottle on ice. After the sample jar was full, the effluent was filtered through a 0.22- μ M cellulose acetate membrane (Corning) to prevent any further biological activity, then placed into a second cleaned glass jar. In the absence of cells, multiple tests showed that filtration does not remove any NDMA from solution. In the presence of cells, filtration was observed to remove a maximum of ~ 7 % of the added NDMA (i.e., due to adsorption or cell uptake). Because of the membrane within the MBR, however, no cells are expected to pass into the effluent water prior to filtration. Methods of NDMA analysis in the influent and effluent are provided in Section 4.4.2.2.

4.4.2.2 Analysis of NDMA in MBR Influent and Effluent

Trace Analysis of NDMA in MBR Effluent by HRMS

After filtration, the ~ 1L effluent samples were stored in amber jars at 4°C, then shipped overnight on ice for extraction and NDMA analysis. Effluent analysis for NDMA was conducted by Maxxam Analytics, Inc (Ontario, Canada) by High Resolution Mass Spectroscopy (HRMS) according to EPA Method 607 Mod 1625 (<http://www.caslab.com/EPA-Methods/PDF/607.pdf>; <http://www.epa.gov/waterscience/methods/method/organics/1625.pdf>). This method, which was designed for trace analysis of NDMA and other nitrosamines, entails a methylene chloride extraction and concentration step, followed by isotope dilution using deuterated NDMA (d6-N-nitrosodimethylamine; d6-NDMA) as a surrogate, and sample analysis by GC/MS/MS. The PQL for NDMA by this method is 2 ng/L. The Maxxam laboratory is certified by the California DHS ELAP program to perform trace NDMA analysis as well as by the Standards Council of Canada, and the Ministry of Environment of Ontario Drinking Water PT Program.

Analysis of NDMA in MBR Influent

The influent water for the MBR did not require NDMA analysis by methods designed for trace (i.e., ng/L) concentrations, since the NDMA in influent water ranged from ~ 10 – 100 µg/L. The influent samples were extracted, concentrated, and analyzed in the Shaw Analytical Testing Laboratory by a modification of EPA Method 8270C (GC/MS) according to the method details described below.

Extraction of NDMA from Influent Water

Extraction of NDMA in the influent samples was conducted by solid phase extraction according to the procedure outlined in EPA Method 521 “Determination of Nitrosamines in Drinking Water by Solid Phase Extraction and Capillary Column Gas Chromatography with Large Volume Injection and Chemical Ionization Tandem Mass Spectrometry (MS/MS)” (http://epa.gov/nerlcwww/m_521.pdf). In summary, a 500 mL volume of influent water was initially amended with d6-NDMA as a surrogate (10 µL of 500 mg/L stock per sample), and then passed through a Resprep solid phase extraction column (Restek, Bellefonte, PA) placed within a Visiprep vacuum manifold (Restek). The NDMA is eluted from the cartridge using 12 mL of methylene chloride. Once eluted, the volume of the methylene chloride was reduced from ~ 12 mL to 1 mL using a Zymark TurboVac II automatic evaporation unit (Caliper Life Sciences, Hopkinton, MA). The residual methylene chloride was then transferred to an amber vial and analyzed for NDMA as detailed in the next section.

GC/MS Analysis of NDMA in MBR Influent Samples

The samples are analyzed for NDMA by a modification of EPA method 8270C “Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS)” (<http://www.caslab.com/EPA-Methods/PDF/8270c.pdf>) using a Hewlett Packard 6890 Series GC fitted with an XTI-5 chromatography column (Restek, Bellefonte, PA; length = 30 m, ID = 0.25 mm, DF = 0.25 µm). The injector was maintained at 240°C and the detector at 250°C. The column was temperature programmed. The initial temperature was 45°C for 2 min., after which the temperature was increased to 225°C at a rate of 20°C and held for 1.0 min. This was followed by a temperature increase to 265 °C at a rate of 35°C and held for 0.5 min. Under these conditions NDMA eluted at ~3.6 min. A Hewlett Packard 5973 Mass Selective Detector was linked to the GC system. The practical quantitation limit (PQL) for NDMA using this method is 0.2 mg/l. The internal standard used for calibration was d14-N-nitrosodimethylpropylamine and, as noted in the extraction section, the surrogate was d6-NDMA. Data was collected using selective ion monitoring (SIM) to increase sensitivity. For NDMA, the ions monitored were 74, 42, and 43. For the surrogate, ions monitored were 80, 35 and 46, and for the internal standard the ions monitored were 46, 48 and 80. All calibrations and QA/QC follow standard procedures as outlined in EPA Method 8270C.

Figure 43. Components of the propane-fed membrane bioreactor (MBR).

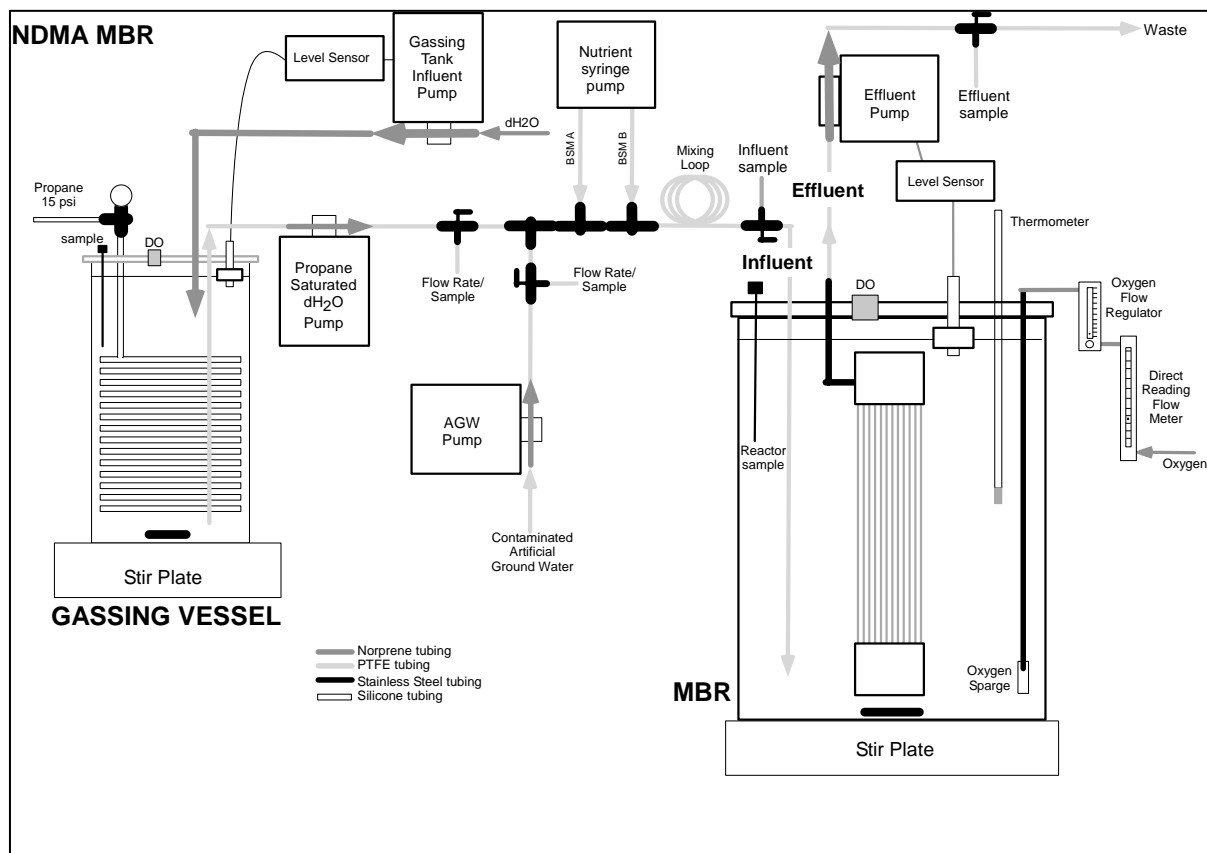


Figure 44. Photograph of the propane-fed membrane bioreactor (MBR).

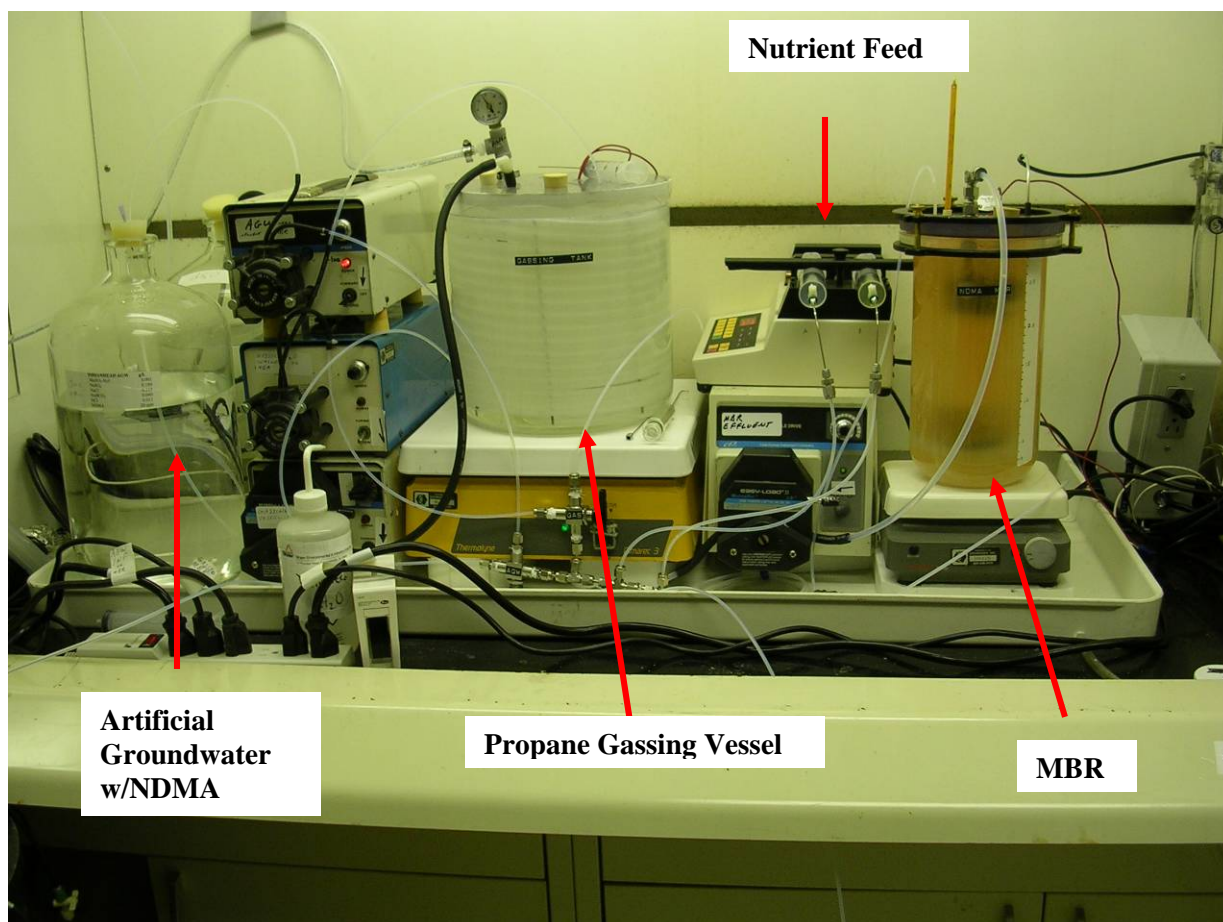
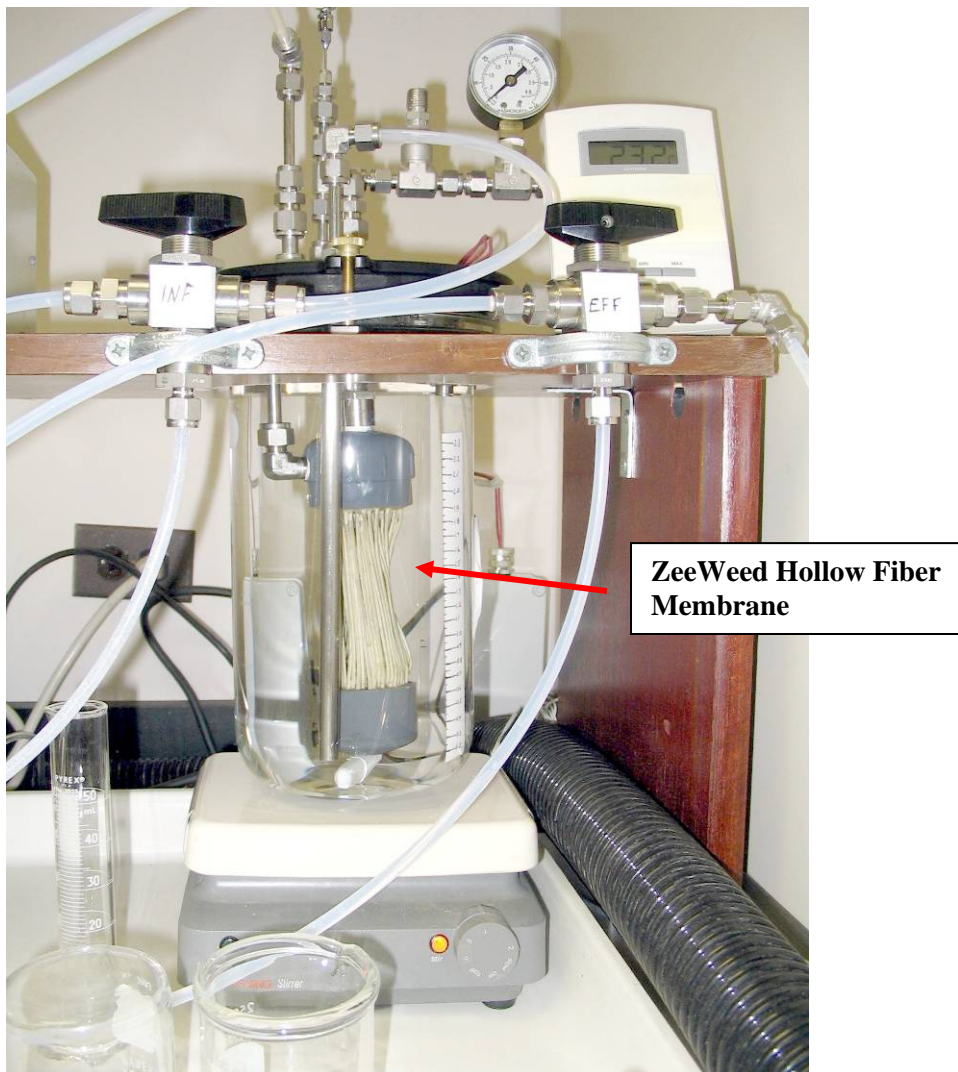


Figure 45. Photograph of the ZeeWeed hollow fiber membrane cartridge in a MBR.



4.4.2.3 Results and Discussion: Propane-Fed MBR

4.4.2.3.1 NDMA

During the 35 days of reactor operation prior to seeding with ENV425, influent and effluent NDMA levels were similar, showing that adsorption and/or volatilization were not significant mechanisms of contaminant loss in the laboratory MBR system (Figure 46; Table 4). Within two days of seeding the reactor with ENV425 (Day 38), effluent NDMA concentrations dropped from $\sim 9.5 \mu\text{g/L}$ to 10 ng/L . These effluent concentrations declined further to 3.1 ng/L after 10 days, and were below detection (i.e., $< 2 \text{ ng/L}$) by 21 days (Figure 46; Table 4). During the initial 75 days of operation after seeding the reactor, with influent levels averaging $7.4 \pm 1.5 \mu\text{g/L}$, NDMA effluent concentrations from the reactor did not exceed 7 ng/L .

The influent levels of the MBR were increased to $\sim 80 \mu\text{g/L}$ on Day 107 of reactor operation. There was a single sample in which an effluent level of $\sim 3 \mu\text{g/L}$ was detected after the influent NDMA concentration was increased (Day 121) (Figure 46; Table 4). After this time, the effluent concentration again decreased to $< 3 \text{ ng/L}$, and remained at or below 10 ng/L through 162 Days (126 days after ENV425 was seeded). The $3 \mu\text{g/L}$ effluent level on Day 121 may have been the result of maintenance work on the reactor that caused disruption of biomass adsorbed to the membrane unit within the MBR. The membrane module was cleaned to improve flux.

The initial 6 months of operational data with NDMA as the sole contaminant clearly indicate that a biological reactor system can achieve effluent NDMA concentrations in the low ng/L range and that these concentrations can be maintained for months of operation. Moreover, the data show that NDMA concentrations in the range normally detected in groundwater at DoD facilities (i.e., $5\text{-}100 \mu\text{g/L}$) are readily treatable in a biological reactor. These laboratory data are very encouraging as there are currently no viable *in situ* or *ex situ* biological treatment options for NDMA.

4.4.2.3.2 Propane and Dissolved Oxygen

The dissolved propane concentration entering the reactor was initially in the vicinity of 20 mg/L (Figure 47). This concentration was increased to $\sim 30 \text{ mg/L}$ after 40 days by increasing the pressure of propane from 10 to $\sim 12 \text{ psi}$ in the propane gassing vessel. The influent propane concentrations averaged $30 \pm 13 \text{ mg/L}$ over the course of reactor operation. The effluent propane levels were generally below detection ($\sim 1 \text{ mg/L}$), although levels of $1\text{-}3 \text{ mg/L}$ were detected on occasion during the first 180 days of reactor operation. Effluent propane concentrations were not taken after the first 6 months. Propane was generally detectable in the headspace of the reactor, although concentrations showed significant variation with time (Figure 48). The propane level in the headspace of the reactor preceding culture inoculation was $\sim 800 \text{ ppmv}$, whereas levels after inoculation averaged $\sim 108 \text{ ppmv}$ ($\pm 165 \text{ ppmv}$).

The dissolved oxygen (DO) in the reactor averaged 10.8 ± 5.8 mg/L during the course of the 380-day study (Figure 49). Throughout the course of testing with artificial groundwater (Day 0-Day 322), the DO was in excess of 5 mg/L on all but a few days. The measured DO within the reactor dropped somewhat within a few weeks of adding the WSTW water. The reason for this drop is unclear, as the oxygen flow into the reactor was accomplished using a sparge stone. The flow from the DO tank to the sparge stone was monitored daily. Thus, the same flow of oxygen was supplied to the reactor. It is possible that the additional consumption of DO reflects usage due to dying biomass within the reactor. The oxygen flow was adjusted upward to account for the reduced DO during this period, and the concentration generally remained > 1 mg/L. Thus, the reactor did not become anoxic.

4.4.2.3.3 Temperature and pH

During the course of the ~ 380 day bioreactor study, the temperature within the MBR averaged $22.6 \pm 1.4^\circ\text{C}$ (Figure 50). The pH of the influent water averaged 8.1 ± 0.5 during the study, while the pH within the MBR was somewhat lower at 6.7 ± 0.3 (Figure 51). The MBR system did not have online pH control, but significant alkalinity was present in the artificial groundwater as well as to the site water to buffer the reactor system.

Figure 46. Influent and effluent concentrations of NDMA in the laboratory MBR during the initial 6 months of operation. The influent levels are given as black triangles and the effluent levels are red circles. The reactor was inoculated on Day 37.

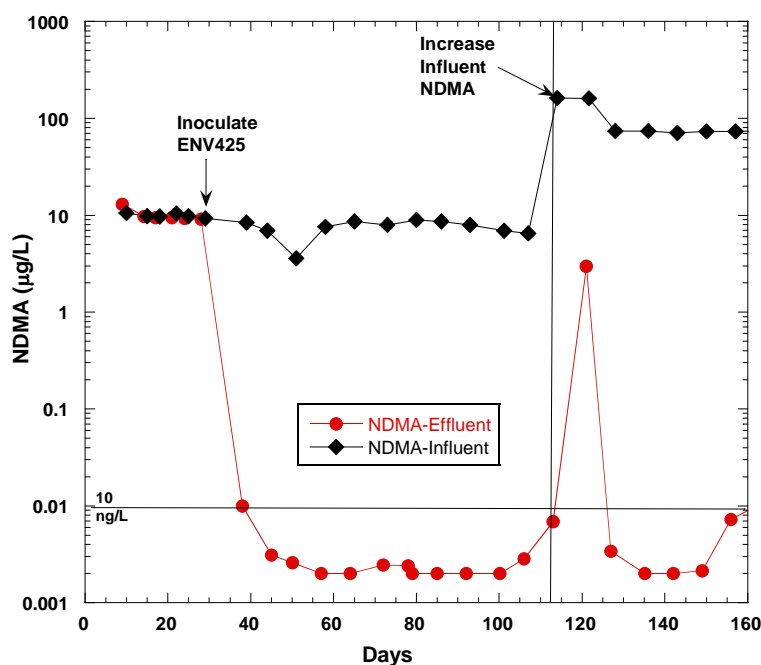


Table 4. Influent and effluent concentrations of NDMA in the propane-fed membrane bioreactor (MBR)

Day of Operation¹	Effluent (µg/L)	Influent (µg/L)
0-1	10	NS ²
9-10	13.0	10.5
14-15	9.7	9.8
17-18	9.4	9.7
21-22	9.4	10.4
24-25	9.3	9.8
28-29	9.1	9.3
36 – Add <i>R. ruber</i> ENV425		
38-39	0.010	8.4
43-44	NS	6.9
45	0.0031	NS
50-51	0.0026	3.6
57-58	< 0.002	7.6
64-65	< 0.002	8.6
72-73	0.0025	7.9
78	0.0024	NS
79-80	< 0.002	8.9
85-86	< 0.002	8.6
92-93	< 0.002	7.9
100-101	< 0.002	6.9
106-107	0.0028	6.5
113	0.0068	NS
114 – Increase NDMA	NS	162.2
121-122	2.96	160.4
127-128	0.0034	74.2
135-136	< 0.002	73.6
142-143	< 0.002	70.9
149-150	0.0021	73.1
156-157	0.0073	73.2
162-163	0.0100	74.9
169-170	0.130	71.6
171 – Begin TCE	NS	NS
177-178	0.116	71.0
183-184	0.0983	69.7
190-191	0.544	72.9
204-205	3.42	27.9

Table 4. Influent and effluent concentrations of NDMA in the propane-fed membrane bioreactor (MBR) (*Continued*)

Day of Operation¹	Effluent (µg/L)	Influent (µg/L)
211-212	12.3	67.4
218-219	16.6	67.1
225-226	15.3	63.5
232-233	11.6	64.3
239-240	27.1	59.3
240-Discontinue TCE	NS ²	NS
247-248	3.35	60.7
253-254	0.541	68.0
260-261	0.505	73.3
267-268	0.066	75.3
275-276	0.060	86.7
281-282	0.035	85.4
288-289	0.045	74.5
295-296	0.039	82.7
303-304	0.055	80.8
309-310	0.105	81.4
322 – Begin WSGW	NS	NS
325	0.0103	10.0
329	NS	10.0
332-333	0.016	10.9
337-339	0.070	10.7
352-353	1.58	9.1
359-360	0.601	10.2
365-366	0.192	9.1
372-373	0.213	9.4
379-380	0.315	7.8

¹ Sample collection requires 10 hr, so influent and effluent samples are collected on consecutive days with the effluent being collected previous to the influent.

² NS: not sampled.

³ NA; not yet available from analytical labor.

Figure 47. Influent and effluent propane concentrations for the laboratory MBR. The influent dissolved propane level is provided as blue squares, the influent propane pressure is given in green triangles, and the effluent propane levels are red circles. The reactor was inoculated on Day 37.

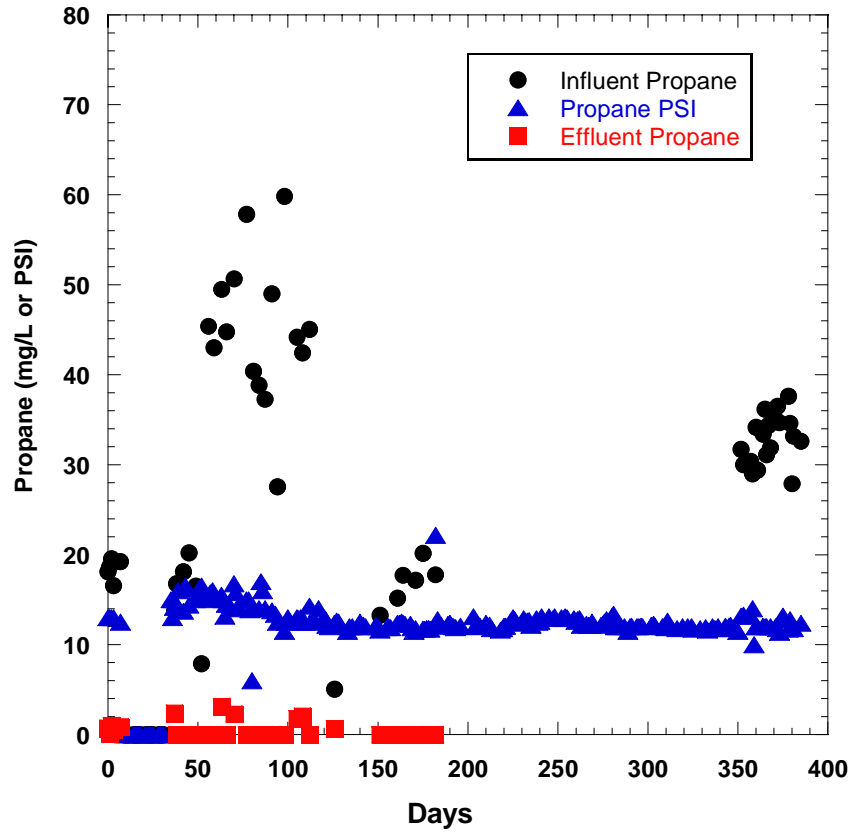


Figure 48. Headspace propane concentrations in the laboratory MBR. The headspace propane levels are provided in ppmv. The reactor was inoculated on Day 37.

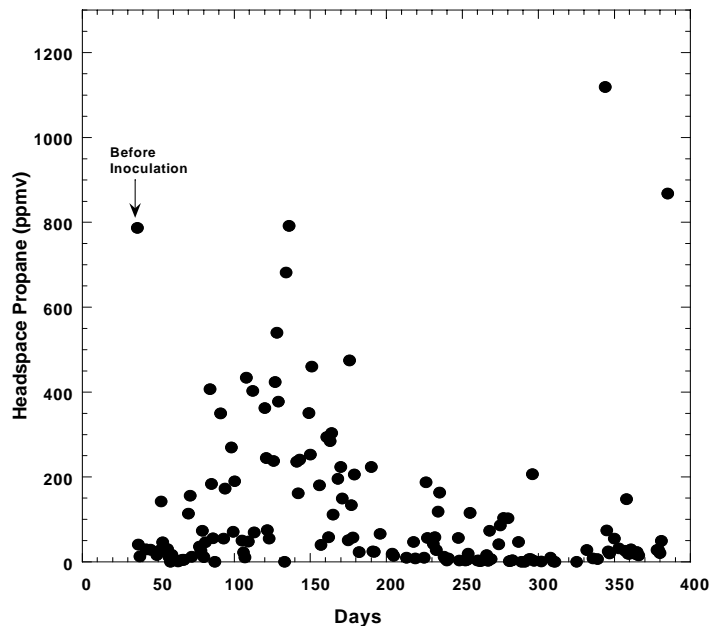


Figure 49. Dissolved oxygen concentrations in the laboratory MBR. The DO concentrations are provided in mg/L. The highest concentration detectable by the DO meter was 20 mg/L. Some values plotted at 20 mg/L may have been somewhat higher.

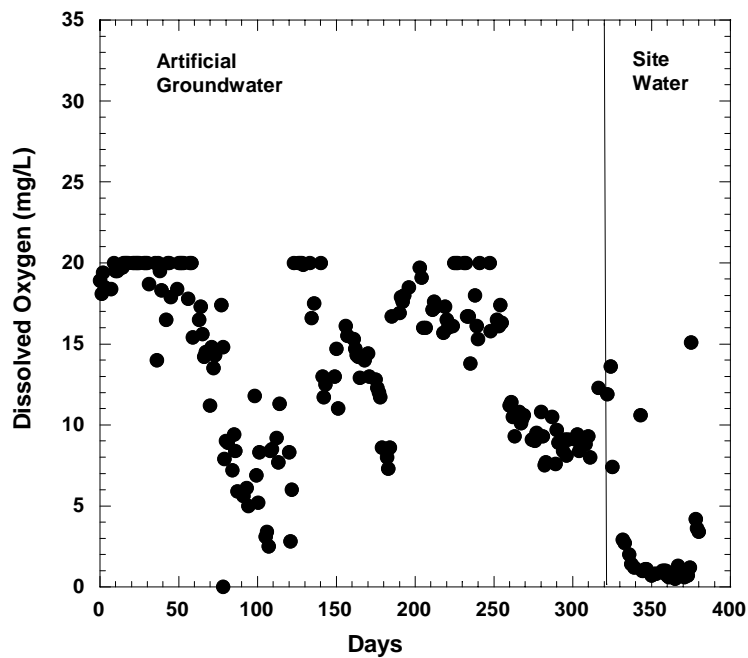


Figure 50. Temperature of the water in the laboratory MBR.

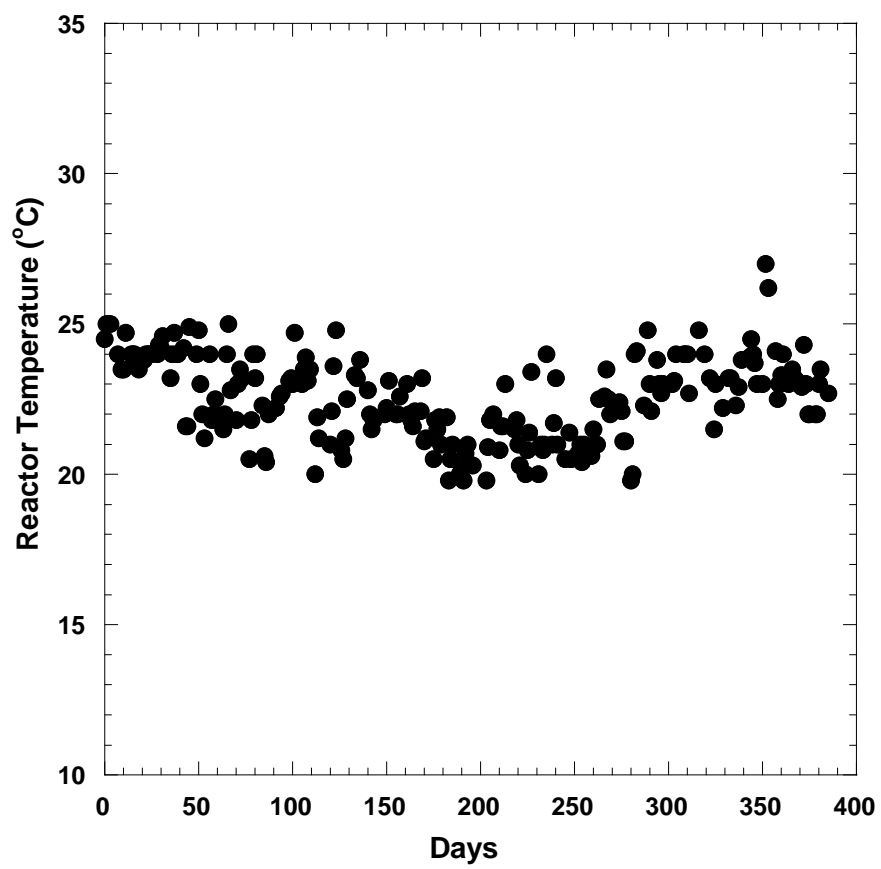
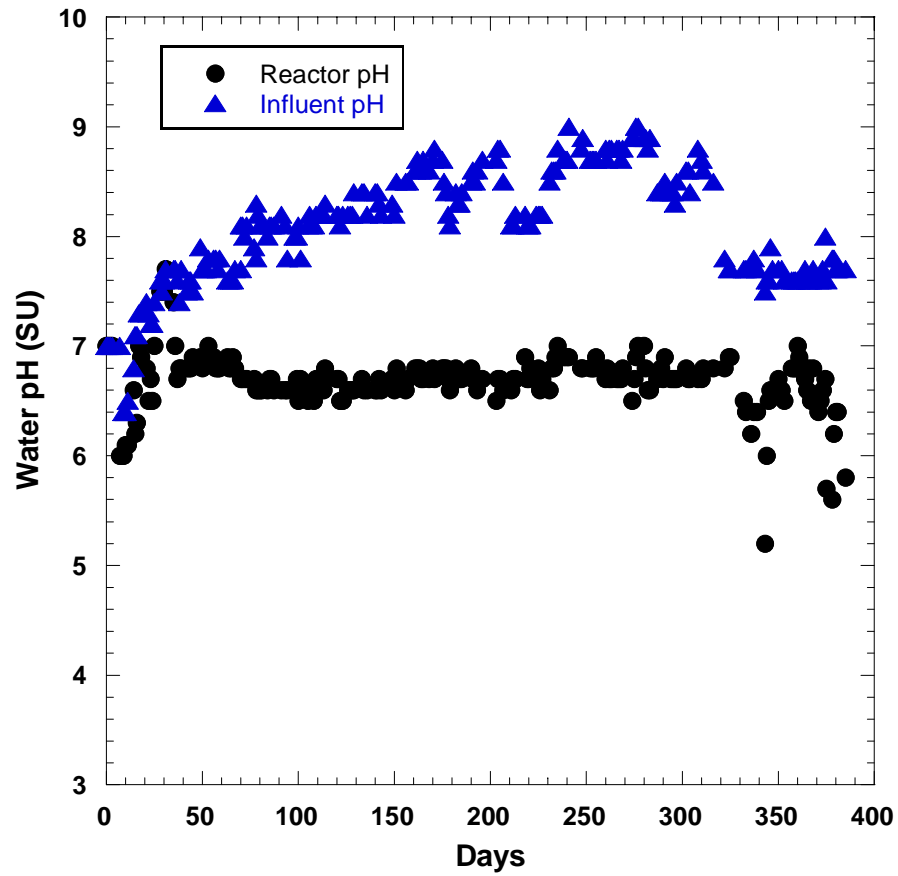


Figure 51. pH of the influent water and the water within the laboratory MBR.



4.4.3 Influence of TCE on NDMA Degradation in a MBR

Trichloroethene (TCE) was introduced to the MBR system as a co-contaminant after the initial several months of successful operation. Prior to adding TCE, a batch study was conducted to determine whether ENV425 was capable of oxidizing the compound. As with several other propanotrophs (e.g., Tovanabootr and Semprini, 1998; Wackett et al., 1989), ENV425 was observed to oxidize TCE after growth on propane (*data not shown*). TCE was supplied to the reactor in the influent groundwater line using a glass syringe and a syringe pump beginning on Day 171 of reactor operation. The influent concentration of TCE was set at ~ 200 µg/L. Influent and effluent TCE samples were collected on a weekly basis in glass GC vials. The TCE was analyzed according to EPA Method 8260 (purge-and-trap with GC-MS). TCE addition was discontinued on Day 240 of reactor operation. Reactor samples were also collected and analyzed for NDMA, propane, pH, and dissolved oxygen as described previously.

The levels of TCE present in the reactor effluent averaged approximately 80 µg/L compared to an average influent level of ~ 200 µg/L (excluding one anomalously high point at Day 204) (Figure 53). Thus, an average decline in TCE of 60% was observed. Obviously, the extent of degradation of TCE within the reactor is appreciable lower than that observed for NDMA, for which effluent concentrations averaged 4-5 log orders lower than the influent concentrations during the initial 5 months of operation of the active reactor. In addition, the percentage of TCE lost to sorption or volatilization is not known because the compound was not added prior to seeding the reactor with ENV425 (i.e., to quantify abiotic processes). Influent and effluent levels of NDMA were shown to be the same prior to seeding with ENV425, suggesting that there was no appreciable abiotic loss of the nitrosamine.

The addition of TCE to the reactor had a significant effect on effluent levels of NDMA (Figure 52; Table 4). Effluent concentrations of NDMA increased from ~ 10 ng/L on Day 162 (prior to TCE addition on Day 171) to 116 ng/L on Day 177, 3.4 µg/L on Day 204, 17 µg/L on Day 218, and 27 µg/L on Day 239. The addition of TCE to the MBR was discontinued on Day 240. The ability of the reactor to recover from this upset was then evaluated (See Section 4.4.4)

The data from this experimental phase clearly indicate that NDMA treatment is adversely impacted by the presence of TCE. These are several possible explanations for this impact, the most probable of which include substrate inhibition and/or cell toxicity. Our previous studies with strain ENV425 and with indigenous organisms in aquifer samples indicate that propane does not appreciably inhibit NDMA metabolism by propanotrophs (e.g., Figure 13). These data are supported by kinetic experiments conducted by Dr. Alvarez-Cohen at UC Berkeley, in which only minimal inhibition of NDMA metabolism of propane was observed for the propanotroph *M. vaccae* JOB5) (*pers. comm. L. Alvarez-Cohen*). It is for this reason that both *in situ* and *ex situ* NDMA treatment by propanotrophs hold great promise for field application. However,

competitive interactions between TCE and NDMA during biodegradation by propanotrophs have not been studied in detail. Presuming that a propane monooxygenase is responsible for both reactions in ENV425 (and this has not yet been proven, partially because a propane monooxygenase has not been cloned), competitive inhibition of TCE on NDMA metabolism is possible.

The second explanation for the impact of TCE on NDMA metabolism by ENV425 is cell toxicity. During metabolism of TCE by monooxygenase enzymes, including methane monooxygenase and cytochrome-P450 enzymes, a short-lived and highly toxic TCE-epoxide is formed (Alvarez-Cohen and McCarty, 1991; Wackett et al., 1989). Based on pure culture studies (see subsequent Section 4.5.1), it is likely that the formation of this epoxide during TCE metabolism by *R. ruber* ENV425 accounts for the declining rates and extents of NDMA degradation observed in the MBR.

The observation that TCE inhibits NDMA destruction in the MBR does not significantly reduce the potential for this technology in the field. Although it would be beneficial to be able to treat both compounds in one unit operation, *ex situ* aerobic co-metabolic treatment of TCE has never proven to be practical or cost-effective. In groundwater streams in which both compounds are present, TCE can be easily removed by either carbon adsorption or air stripping prior to the reactor system. The latter process has the added benefit of oxygenating the groundwater prior to entering an NDMA bioreactor. As an example, Aerojet (Sacramento, CA) has a 5,000 GPM treatment system for combined nitrate, perchlorate, TCE, and NDMA. The three compounds are removed in sequence by anaerobic biological treatment in fluidized bed reactors (nitrate and perchlorate), air stripping (TCE), and UV treatment (NDMA). In this case, the replacement of the UV system with a more cost-effective approach would be desirable.

4.4.4 Recovery of MBR Performance after Removal of TCE from Influent

4.4.4.1 Materials and Methods: MBR Recovery after TCE Removal

The addition of TCE to the reactor influent water at ~ 200 µg/L was discontinued on Day 240. The other reactor operating conditions remained the same as described for Day 114-170, prior to TCE addition. In summary, the influent flow to the reactor was ~ 100 mL/hr (30 hr HRT), the temperature averaged ~ 22.5°C, and the propane feed pressure was ~ 12 psi (equating to ~ 30 mg/L propane) in solution.

4.4.4.2 Results and Discussion: MBR Recovery after TCE Removal

As previously noted, the reactor effluent data clearly indicate that NDMA treatment was adversely impacted by the presence of TCE. It is probable that substrate inhibition (i.e., TCE inhibiting NDMA metabolism), cell toxicity from TCE-epoxide formation, or some combination of these two factors is responsible for the impact of TCE on NDMA biodegradation. After TCE was removed from the influent, the concentration of NDMA

in the reactor effluent declined rather rapidly (Figure 52; Table 4). The NDMA concentration in the reactor effluent reached a high value of 27.1 $\mu\text{g/L}$ on Day 239, at which time the TCE feed was discontinued. Within ~ 4 weeks (Day 267), the effluent NDMA concentration was down to 66 ng/L, and after 6 weeks (Day 281) the effluent concentration reached 35 ng/L (Figure 52; Table 4). Thus, recovery of reactor performance was rapid considering that the reactor has a HRT of ~ 30 hrs. Effluent levels below 10 ng/L were not achieved before switching the reactor influent to another water source (see next section). However, it is not clear if this related to a residual effect of TCE addition or an increased necessity for reactor membrane cleaning. After operation for more than 10 months, and increased levels of biomass in the reactor, the reactor membrane required cleaning and backwashing on a weekly or twice-weekly basis to remain operational. The cleaning procedure, which includes manually removing biomass from a portion of the hollow-fiber membrane, may have affected reactor performance, resulting in slightly elevated effluent NDMA concentrations.

Figure 52. Influent and effluent concentrations of TCE in the laboratory MBR. The addition of TCE began on Day 171.

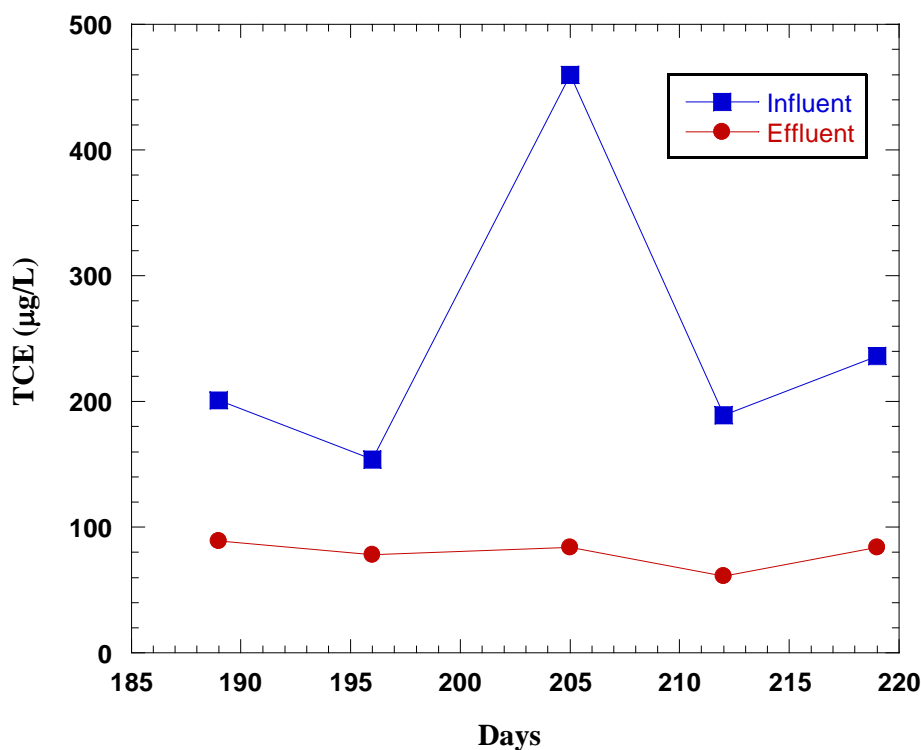
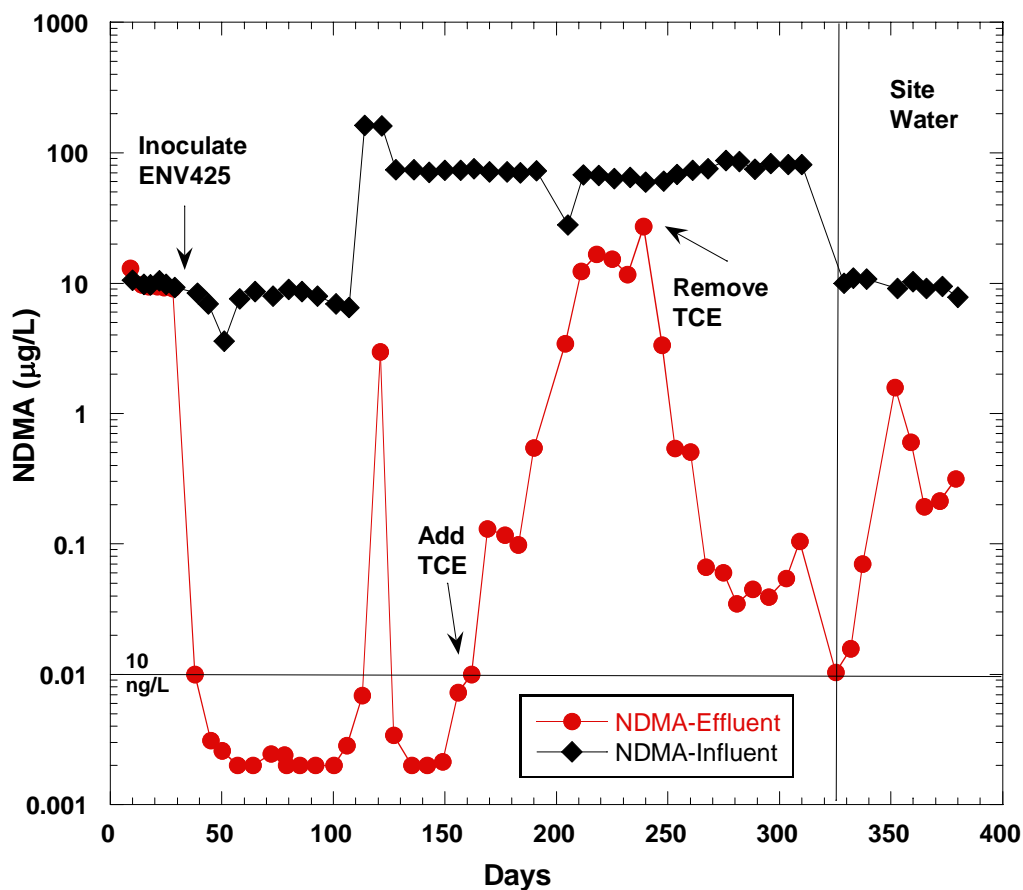


Figure 53. Influent and Effluent concentrations of NDMA in the laboratory MBR. The concentrations are given on a logarithmic scale. The MBR was cleaned of biomass on Day 121 resulting in a temporary increase in NDMA effluent levels. TCE was added at ~ 200 µg/L from Day 171 – 240. Influent feed was changed to groundwater from White Sands, NM on Days 322 - 380.



4.4.5 Treatment of Site Groundwater in a MBR

4.4.5.1 Materials and Methods: Treatment of Site Groundwater

The final phase of reactor operation is an evaluation of performance using actual groundwater from an NDMA-contaminated site. Previous operation utilized an artificial groundwater with NDMA added. For this phase, which began on Day 322 of reactor operation, groundwater was obtained from Well BLM-15 the NASA White Sands Text Facility (WSTF) near Las Cruces, NM. The NDMA concentration in groundwater from this well was ~ 20 µg/L, which results in a 10 µg/L influent concentration to the reactor (the groundwater is mixed 50:50 with propane-saturated water prior to entering the reactor). WSTF is interested in both *in situ* and *ex situ* treatment approaches for NDMA.

The groundwater at WTSF is predominantly aerobic and neutral pH. Bicarbonate concentrations range from 89 to 376 mg/L. Major cations include calcium (22 to 179 mg/L), magnesium (12.6 to 84 mg/L), and sodium (28 to 500 mg/L). Sulfate concentrations range from 185 to 600 mg/L, nitrate is generally less than 10 mg/L, and total iron concentrations range from trace amounts to 0.8 mg/L. Total dissolved solids concentrations in WTSF groundwater range from 490 to 1,230 mg/L. In addition to NDMA, some wells also contain low concentrations of TCE, Freons 13 and 121, and N-nitrodimethylamine. The well chosen for sampling has groundwater chemistry typical of the WTSF site.

4.4.5.2 Results & Discussion: Treatment of Site Groundwater

The WTSF groundwater was fed to the reactor for approximately 2 months beginning on Day 322 of operation (Day 322-Day 380). The addition of this water caused the reactor pH to decline to ~ 6.0, so a bicarbonate solution was added to the WTSF water each time the reservoir was refilled. The average influent concentration during this period was 9.7 µg/L (n = 8), and the effluent ranged from 0.010 to 1.6 µg/L (Figure 53; Table 3). As shown in Figure 53, the effluent from the reactor, increased from 0.016 µg/L on Day 332 to 1.6 µg/L on Day 352. During this interval (Day 339-342), influent water was not supplied to the reactor due to a pump malfunction, so the biomass within the reactor received no propane or nutrients (oxygen was supplied separately, so the reactor did not become anaerobic). The lack of propane addition likely accounted for the increased effluent concentrations during this period. After this time, the effluent levels declined to ~ 0.2 – 0.3 µg/L. It should also be noted that the reactor membrane required frequent cleaning during the period when the WTSF groundwater was supplied due to excessive biomass growth. This cleaning process, although necessary to allow permeate through the membrane, may have negatively affected system performance. The MBR was shut down after the evaluation of NDMA treatment in the WTSF water was complete. An inventory of biomass within the reactor was taken by removing all adsorbed biomass from the membranes and reactor vessel surface, drying the biomass overnight at 110°C, and then weighing. A total of 8.2 g of biomass was present in the reactor after more than 1 yr of operation.

4.5 Influence of TCE and Other Co-Contaminants on NDMA Biodegradation: Pure Culture Studies.

A negative impact of TCE on propane monooxygenase activity (i.e., the enzyme presumed to be responsible for NDMA and TCE oxidation) may account for the observation that TCE significantly inhibited NDMA removal in the MBR study. During this experiment, rates of propane utilization by ENV425 were evaluated after the strain was incubated with various concentrations of TCE. A reduction in rates of propane consumption after exposure to TCE is indicative of enzyme toxicity.

4.5.1 Effect of TCE on Propane Utilization by ENV425

4.5.1.1 Materials and Methods: Effect of TCE on Propane Utilization by ENV425

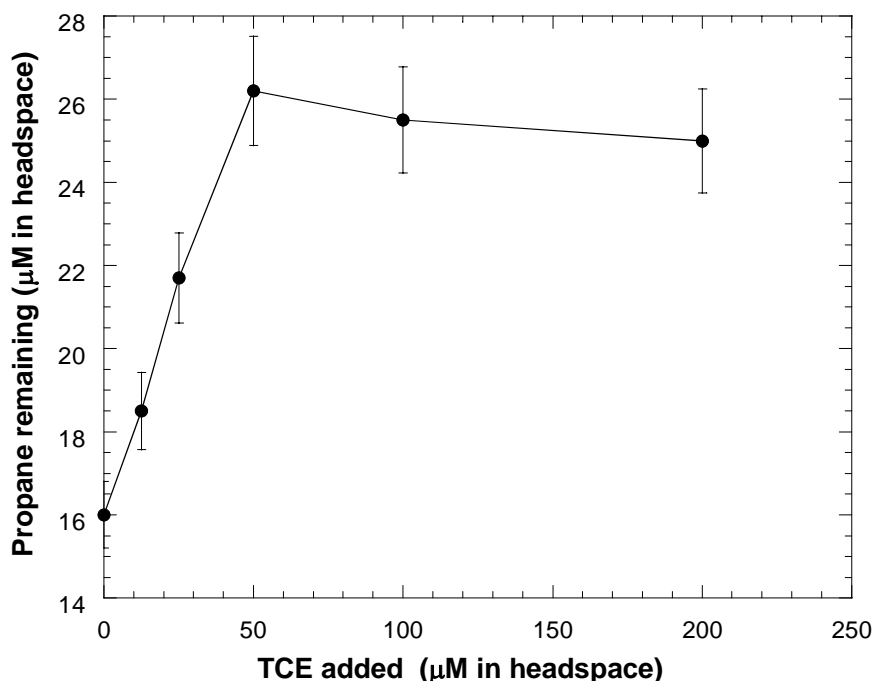
Strain ENV425 was grown in BSM-H with ~25% propane in the headspace. The culture was harvested during logarithmic growth phase, washed, and resuspended in BSM to an OD₅₅₀ of 2.0. Cells were distributed in 5-mL aliquots into 27-mL serum vials and sealed with Teflon lined septa. TCE from a concentrated stock solution was injected into the vials through the septa to achieve final headspace TCE concentrations ranging from 0-200 μ M. Vials were incubated at 30°C with gentle shaking (~70 rpm). The TCE concentrations in the vials that received the highest initial concentrations (100 and 200 μ M, respectively) were monitored periodically via GC analysis of the headspace gas. After ~3 hr, the TCE concentration in these samples was below detection. At this time, propane was added (25 μ M in headspace) to all samples, and the samples were incubated for 1 hr. The propane concentration in the headspace of each sample was then measured by GC.

4.5.1.2 Results and Discussion: Effect of TCE on Propane Utilization by ENV425

The results of this experiment demonstrate that TCE is toxic or otherwise inhibits the oxygenase enzyme responsible for propane utilization in ENV425. Cultures incubated with TCE concentrations of 50 μ M and higher lost their ability to degrade propane (Figure 54). Conversely, ENV425 cells that were exposed to TCE levels of 25 μ M or lower were still able to metabolize propane, although the degradation rates appeared to be reduced compared to controls without TCE.

The impact of TCE on NDMA metabolism by ENV425 is likely to be caused by cell toxicity. During TCE oxidation by monooxygenase enzymes, a transient, but highly toxic TCE-epoxide is formed (Alvarez-Cohen and McCarty, 1991; Wackett et al., 1989). The formation of this epoxide during TCE degradation by *R. ruber* ENV425 is likely to account for the reduced rate of propane metabolism by the strain after incubation with TCE, as well as the reduced rate and extent of NDMA degradation observed previously in the MBR.

Figure 54. Influence of TCE on propane utilization by ENV425. Propane-grown cells were incubated with TCE at the given concentration until all TCE was degraded (~ 3 hr). Propane was then added to all vials at 25 μM in headspace. After 1 hr, the propane remaining in headspace was determined.



4.5.2 Biodegradation of TCE and NDMA by *P. mendocina* KR1

4.5.2.1 Materials and Methods: Biodegradation of TCE and NDMA by KR1

A study was conducted to evaluate the impact of TCE and NDMA biodegradation by the toluene-oxidizer *P. mendocina* KR1. This study was designed to determine whether there would be significant competitive inhibition between the two contaminants, as both substrates of the enzyme toluene-4-monooxygenase (T4MO; Fournier et al., 2006). The study also provides perspective on whether joint treatment of both NDMA and TCE is possible using strain KR1.

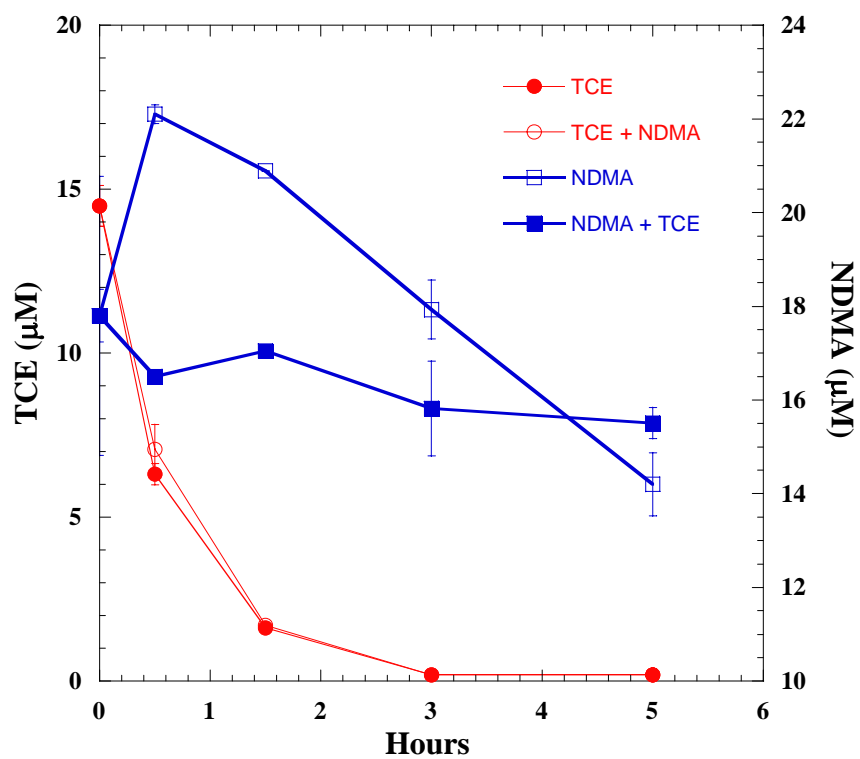
P. mendocina KR1 was grown in shake flasks with vapor phase toluene to an OD_{600} of 1.8, washed, then resuspended in 30 ml of BSM-K (nitrogen free) to an OD_{600} of 1.0. Test conditions consisted of NDMA only (~ 15 μM was desired but the initial concentration was ~ 22 μM); TCE only (~ 15 μM); TCE + NDMA (~ 15 μM TCE and 18 μM NDMA); and killed controls (~ 15 μM TCE and 18 μM NDMA + 2.5% (v/v) formaldehyde). The bottles were shaken in the dark on a rotary shaker at room temperature (22°C).

Duplicate bottles from each treatment were removed from the shaker at 0.5, 1.5, 3, and 5, and 24 h after initial set-up. Subsamples (8 mL) were preserved with HCl, and analyzed for TCE by EPA method 8260 (GC/MS). Bottles with NDMA were centrifuged for 5 min to remove cells, and then the supernatant was extracted with an equal volume of methylene chloride (CH_2Cl_2) by shaking (150 rpm overnight). After a period of sonication to promote phase separation, the CH_2Cl_2 was removed by glass pipette and passed through sodium sulfate (NaSO_4) to remove residual water. The extract was concentrated to a 1 mL final volume using a solid-phase extraction vacuum manifold (Visiprep DL, Supelco, Bellefonte, PA). NDMA analysis was performed on the concentrated sample by GC-MS using EPA method 8270 modified to use SIM (single ion monitoring) as detailed in Section 4.4.2.2.

4.5.2.2 Results and Discussion: Biodegradation of TCE and NDMA by KR1

TCE was rapidly degraded by strain KR1 in the presence or absence of a nearly equimolar quantity of NDMA (Figure 55). The initial 15 μM of TCE was below detection ($< 0.2 \mu\text{M}$) within 3 hr in the presence or absence of NDMA. The maximum rate of degradation of TCE in the presence or absence of NDMA was approximately 15 $\mu\text{M/hr}^{-1}$. Thus, NDMA does not appear to inhibit TCE oxidation by *P. mendocina* KR1 at the concentrations tested. When present individually, the rate of NDMA metabolism by strain KR1 was considerably slower than observed for TCE. The maximum rate of degradation was approximately 1.8 $\mu\text{M NDMA/hr}^{-1}$. It should be noted that the rate is calculated using data from the 0.5 – 5 hr time points. NDMA transformation by the organism was much slower during the initial 5 hr in samples also receiving TCE, with a maximum rate of only 0.3 $\mu\text{M NDMA/hr}^{-1}$. At the end of 24 hr, NDMA was degraded to $< 2 \mu\text{M}$ in bottles with or without TCE added, but no losses were apparent in formaldehyde-killed controls (data not shown). The data further confirm that T4MO is the enzyme responsible for NDMA oxidation, and suggest that TCE is preferentially oxidized when both substrates are present. The data also show that degradation of both NDMA and TCE by this strain is possible.

Figure 55. Degradation of NDMA and TCE by *P.mendocina* KR-1. Toluene-grown cells were incubated with TCE, NDMA, or TCE + NDMA, and concentrations of both compounds were measured with time.



4.6 Evaluate Potential for Treatment of NDMA with Cosubstrates and Bioaugmentation

4.6.1 Influence of Cosubstrates on NDMA Mineralization in Aquifer Microcosms

The effectiveness of propane and other co-substrates to stimulate naturally-occurring NDMA-degraders was evaluated in traditional aquifer microcosms (i.e., groundwater plus aquifer solids). Previous studies conducted during this project have indicated that NDMA biodegradation in nature is largely a co-metabolic process, with exogenous organic carbon required for biological activity to occur. In this series of experiments, aquifer samples were incubated with one of three organic substrates for three weeks (propane, yeast extract, or lactate), then the samples were amended with NDMA, and mineralization of the nitrosamine was measured and compared to unamended control samples. The application of bioaugmentation for NDMA treatment in samples from the PJKS site in Colorado was also examined.

4.6.1.1 Materials and Methods: NDMA Mineralization with Cosubstrates

Sterile serum bottles received 25 g of homogenized aquifer sediments and 25 mL of groundwater from one of three sites; New Jersey, southern California, or Air Force Site PJKS. Triplicate bottles then received one of the following amendments: (1) 100 mg/L lactate, (2) 3% propane in bottle headspace, (3) 100 mg/L yeast extract or (4) no carbon addition. Each of the bottles receiving organic carbon also was amended with 0.55 ml of a solution containing 10 g/L $(\text{NH}_4)_2\text{HPO}_4$ and 5 g/L $\text{NH}_4\text{H}_2\text{PO}_4$ as a source of inorganic nutrients. The microcosm bottles were sealed with Teflon-lined septa and placed on a shaker operating 100 rpm at room temperature (22°C). After 5 days and 15 days of shaking, each bottle received another addition of each organic amendment (nutrients were not added subsequently). After 20 days of incubation, each bottle received 50 µg/L of NDMA and approximately 50,000 dpm of ^{14}C -NDMA. A base trap containing 1 ml of 0.5N NaOH was also added to each bottle. All bottles were placed at room temperature and shaken at 100 rpm. The NaOH in each trap was sampled periodically to assess mineralization of $^{14}\text{CO}_2$. The liquid organic amendments (lactate and yeast extract) were added to each bottle on a weekly basis, and propane was re-supplied each time the bottles were sampled.

4.6.1.2 Methods: Bioaugmentation for NDMA Treatment.

Microcosms were prepared from site PJKS as described in the previous section (i.e., 25 g of site sediments and 25 g of groundwater). Samples were then amended with the following: (1) 20 mL of a mixture of 75% propane and 25% oxygen in headspace and strain ENV425 ($\text{OD}_{550} = 1$), (2) 20 mL of a mixture of 25 % propane and 75% oxygen in bottle headspace with no culture, (3) 100 mg/L lactate, (4) no carbon addition or (5) formaldehyde (2 %) as a killing agent. Each bottle then received 50 µg/L NDMA and 50,000 DPM ^{14}C -NDMA. A base trap was also added to each bottle, and traps were

sampled and analyzed for $^{14}\text{CO}_2$ as described previously. Twenty mL of the mixture of 25% propane and 75% oxygen was added to the headspace of bottles in Treatment 1 and Treatment 2 each time the NaOH was sampled. Bottles were incubated in the dark on a shaker (100 rpm) at room temperature.

4.6.1.3 Results and Discussion: NDMA Mineralization with Cosubstrates

The addition of propane to site samples prior to adding NDMA caused a marked stimulation in the subsequent mineralization of the nitrosamine in each set of site aquifer samples (Figures 56-58). These data suggest the following: (1) that indigenous propanotrophs in many groundwater aquifers are capable of NDMA metabolism; (2) that the process is co-metabolic and independent of the presence or absence of NDMA (i.e., these organisms were enriched on propane only, but were readily capable of NDMA mineralization; and (3) that propane addition may be a viable *in situ* remedial approach for NDMA bioremediation of groundwater aquifers. Interestingly, yeast extract also stimulated NDMA biodegradation in each of the three sets of samples whereas lactate was ineffective. Apparently, much like propane, yeast extract also stimulates a species or consortium of microorganisms capable of degrading NDMA. Additional studies are required to assess which organisms are degrading NDMA as a result of yeast extract amendment.

NDMA mineralization occurred in unamended samples (~ 10-15% over 35 days), but the rates and extents were appreciably less than observed in the propane and yeast extract treated microcosms. Greater extents of mineralization of NDMA have been observed in previous studies with unamended samples, particularly from the southern California and New Jersey locations. However, we have previously hypothesized that this activity is supported largely through co-metabolic degradation of natural organic matter. Moreover, studies using extracted organic matter have supported this hypothesis (see Figure 26a). In this study, pre-incubating samples under aerobic conditions for three weeks prior to NDMA addition is likely to have resulted in the degradation of most bioavailable organics. Thus, these materials were not present to support co-metabolic biodegradation of NDMA.

4.6.1.4 Results and Discussion: Bioaugmentation for NDMA Treatment.

The addition of propane and *R. ruber* ENV425 to site samples from PJKS caused the most rapid mineralization of NDMA among the treatments tested (Figure 59). No lag phase was observed, and more than 60% of the added NDMA was mineralized in 7 days. By comparison, very little NDMA mineralization was observed in microcosms receiving propane (but no culture) during the initial 2 weeks of incubation. It should be noted that unlike the microcosms in Figures 56-58, these samples were not pre-incubated with propane prior to adding NDMA. After 2 weeks, NDMA mineralization was observed in the PJKS samples receiving propane, and by 50 days, nearly 70% of the added NDMA

was collected as $^{14}\text{CO}_2$. (Figure 59) The data from this study show that bioaugmentation with an exogenous propanotroph can enhance rates of NDMA mineralization in PJKS aquifer samples. However, with additional time, indigenous bacteria in the aquifer will be stimulated to degrade NDMA if propane is added. These data support the findings presented for Site PJKS in Figure 58.

Most importantly, the data presented from this series of studies show that *in situ* bioremediation of NDMA through propane (and oxygen) addition may be a viable approach for NDMA treatment in groundwater aquifers. Other organic substrates, such as yeast extract, may also be useful for NDMA bioremediation if aerobic conditions are maintained. The levels of NDMA that indigenous propanotrophs can achieve *in situ* was evaluated in large-scale microcosms (see Section 4.6.2). It was previously unclear whether any bacterium could reduce NDMA to ng/L levels. However, both reactor and batch data from this SERDP project clearly show that the propanotroph ENV425 can reduce NDMA levels from ~ 50 µg/L to below 2 ng/L. Moreover, microcosm studies conducted with aquifer samples collected from 2 different locations suggest that low ng/L concentrations may also be attained by indigenous propanotrophs in these environments (see Section 4.6.2). In addition, the impact of common co-contaminants, particularly chlorinated solvents, on the effectiveness of propane biostimulation for NDMA treatment is an important consideration. It is possible that TCE and other solvents (which are also substrates for oxygenase enzymes expressed by propanotrophs) may inhibit *in situ* biodegradation of NDMA. However, data from microcosm studies conducted during this project suggest that, at least for some of the sites evaluated, both TCE and NDMA can be treated via propane biostimulation (see Section 4.6.3). Thus the data from this study provide significant evidence that *in situ* NDMA treatment via propane addition may be a viable option for groundwater treatment.

Figure 56. Mineralization of ^{14}C -NDMA in carbon-amended aquifer microcosms from New Jersey. The microcosms received propane (3%), yeast extract (100 mg/L), lactate (100 mg/L) or no addition, and were incubated for three weeks prior to NDMA addition. Bottle received periodic addition of each amendment after NDMA was added.

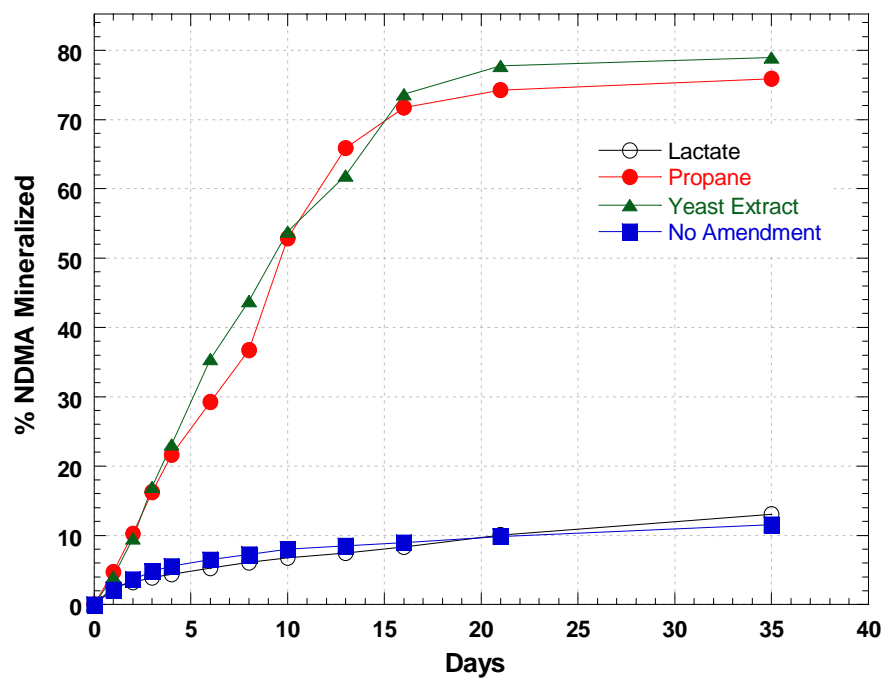


Figure 57. Mineralization of ^{14}C -NDMA in carbon-amended aquifer microcosms from southern California. The microcosms received propane (3%), yeast extract (100 mg/L), lactate (100 mg/L) or no addition, and were incubated for three weeks prior to NDMA addition. Bottle received periodic addition of each amendment after NDMA was added.

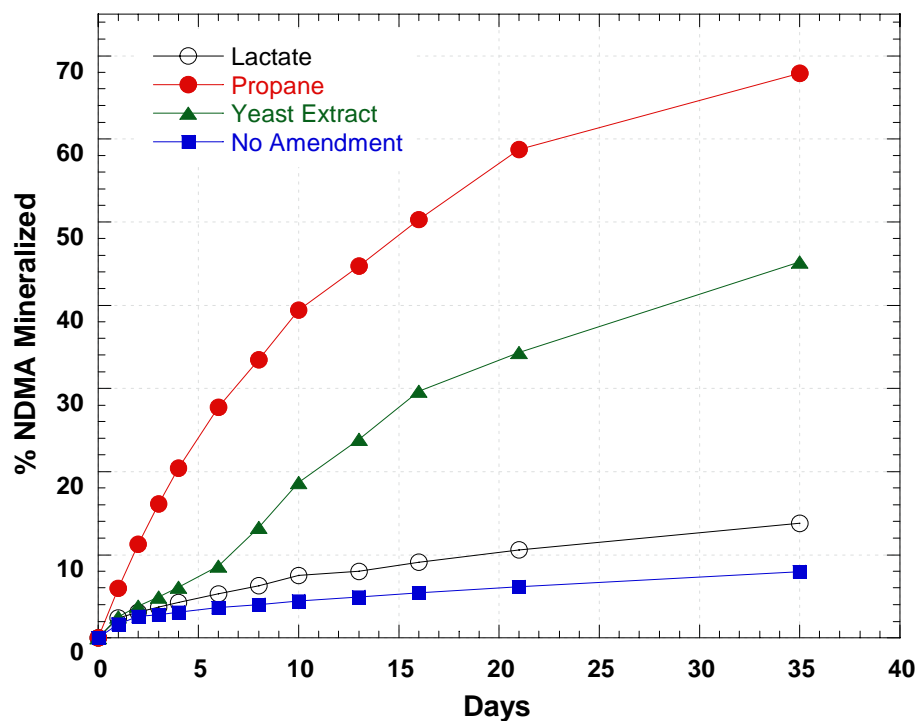


Figure 58. Mineralization of ^{14}C -NDMA in carbon-amended aquifer microcosms from Air Force Site PJKS, CO. The microcosms received propane (3%), yeast extract (100 mg/L), lactate (100 mg/L) or no addition, and were incubated for three weeks prior to NDMA addition. Bottle received periodic addition of each amendment after NDMA was added.

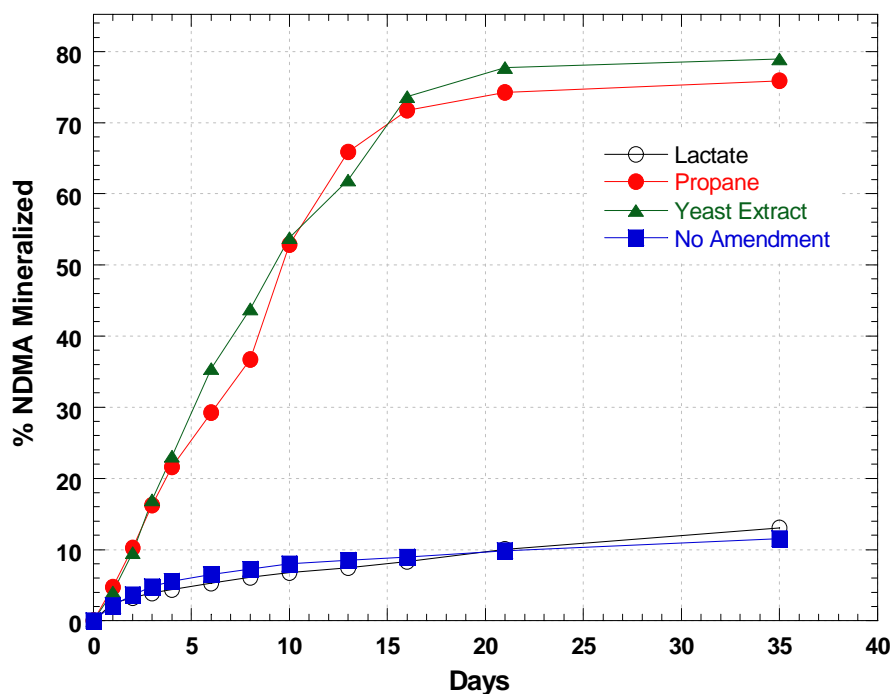
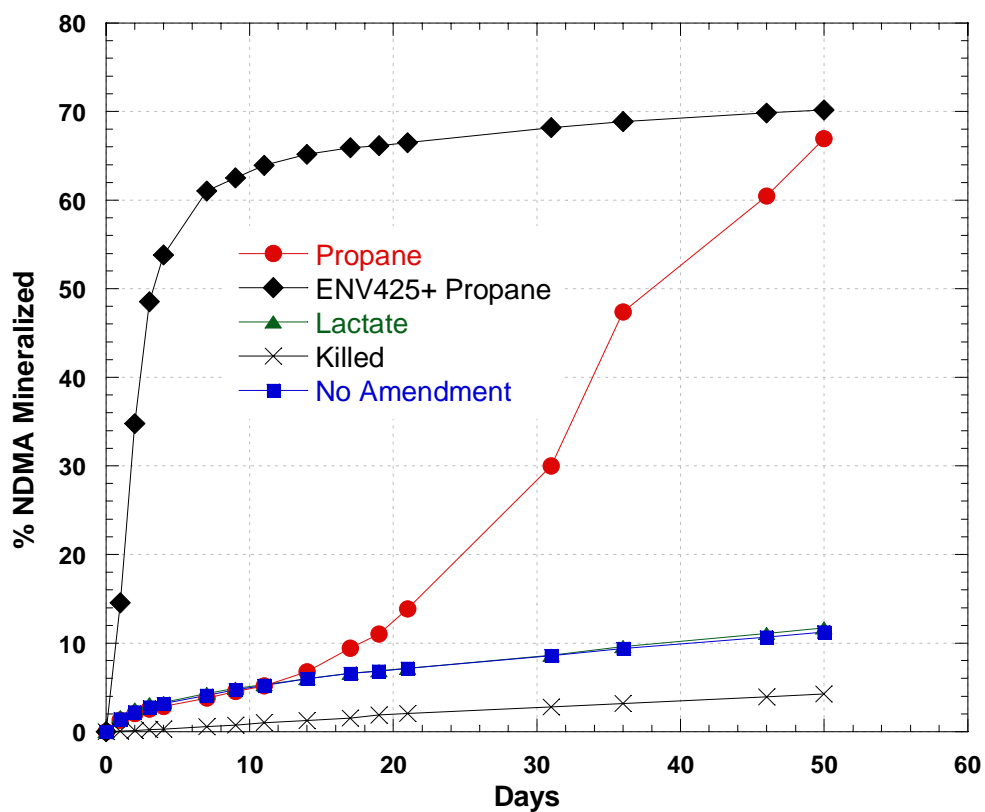


Figure 59. Influence of biostimulation and bioaugmentation on the mineralization of ^{14}C -NDMA in aquifer microcosms prepared from Air Force Site PJKS aquifer materials. The microcosms received *R. ruber* ENV425 + propane (25%), propane only (25%), lactate (100 mg/L) or no addition. Killed control samples were treated with 2% formaldehyde.



4.6.2 *In Situ* Treatment of NDMA using Propane Biostimulation

4.6.2.1 *Background and Methods: Propane Biostimulation*

An important objective of this SERDP project is to determine whether *in situ* degradation of NDMA to low ng/L concentrations is possible via propane biostimulation. A study was designed to meet this objective. Large volumes of groundwater were needed for this experiment because analysis of NDMA at ng/L levels requires ~ 0.5 to 1 L of sample. Eight microcosms were prepared in clean 4L glass sample jars. Each jar received 300 g of homogenized aquifer solids and 3 L of mixed site groundwater (Figure 60). The lids of the jars were drilled, and barb fittings were placed in each to allow for gas addition (i.e. oxygen, propane). Four microcosms received site materials from New Jersey, and four received site materials from Air Force Plant PJKS, CO. The headspace of all of the microcosms was flushed with oxygen, then three of the four microcosms from each site received a mixture of 80% oxygen and 20% propane gas. A Tedlar bag containing this gas mixture was attached to the lid of each microcosm to ensure an adequate supply of propane and oxygen. One microcosm from each site (no-propane control) received only oxygen gas in the headspace. Nutrients (60 mg/L nitrogen and 80 mg/L phosphorus) were added bi-weekly to ensure that a limitation in nitrogen and/or phosphorus did not occur. Bottles were shaken at room temperature (23-25°C) for 28 days. The headspace in each bottle and Tedlar bag was replaced once per week.

After 4 weeks of pre-incubation with propane, 10 mL of groundwater was removed from one propane treated microcosm and one control microcosm from each site and placed into a 160-mL serum bottle. Unlabeled NDMA (100 µg/L) and a small amount of radiolabeled NDMA were added to each groundwater sample along with a Durham tube containing 0.5 mL of 0.5 N NaOH. The production of $^{14}\text{CO}_2$ from the ^{14}C -NDMA indicated that pre-incubation with propane had stimulated activity in each set of samples. Unlabeled NDMA was subsequently added to the large microcosm bottles to an initial concentration of ~ 100 µg/L. At this time, 160-mL of groundwater was removed from one experimental jar from each site and replaced with an equal volume of a 37% formaldehyde stock solution (final formaldehyde concentration = 2 % v/v); these jars served as killed controls. Microcosms were allowed to shake for 90 min to allow the NDMA to mix throughout the microcosm, then 500 mL of groundwater (exact sample volume determined by weight) was removed from each microcosm and filtered through a 0.2 micron filter. Each microcosm was then incubated in the dark at room temperature (~ 23°C). Headspace gas, as described for the pre-incubation period, was replaced on a weekly basis, and 500-mL samples were collected weekly (New Jersey) or bi-weekly (PKJS). The samples were filtered, then submitted to Maxaam Analytical for low level NDMA analysis by GC/MS/MS as previously described for reactor effluent samples (Section 4.4.2.2).

4.6.2.2 Results and Discussion: Propane Biostimulation

NDMA was reduced from high ppb levels (80 to 100 $\mu\text{g/L}$) to low ppt levels within one week in all test microcosms that had received propane in the headspace. In New Jersey microcosms, NDMA levels as low as 8 to 9 ng/L were obtained within 1 week, and levels were as low as 5 ng/L within 2 weeks (Figure 61). In PJKS microcosms, NDMA levels were reduced to 7 to 15 ng/L within 1 week (Figure 62). At 2 weeks some microcosms showed a slight increase in NDMA, which might be due to desorption of NDMA from the sediment as it is degraded in the groundwater. These increases were minor and never exceeded 37 ng/L in any of the test microcosms.

The results from these microcosms are important because they (1) confirm that propane-oxidizing bacteria capable of degrading NDMA are present in some groundwater aquifers, and (2) reveal for the first time that these organisms can be stimulated to biodegrade NDMA from typical groundwater concentrations at military sites (i.e., 10-100 $\mu\text{g/L}$) to low ng/L concentrations. These data suggest that *in situ* treatment of NDMA via propane biostimulation may be a viable remedial option at military and NASA facilities that have this contaminant in groundwater.

Figure 60. Photograph of large-scale microcosms. Tedlar bags contain oxygen or a mixture of oxygen and propane gas.



Figure 61. Biodegradation of NDMA in laboratory microcosms prepared with aquifer solids and groundwater from the New Jersey site. The microcosms were pre-incubated with oxygen and propane, and then spiked with NDMA to a starting concentration of $\sim 100 \mu\text{g/L}$.

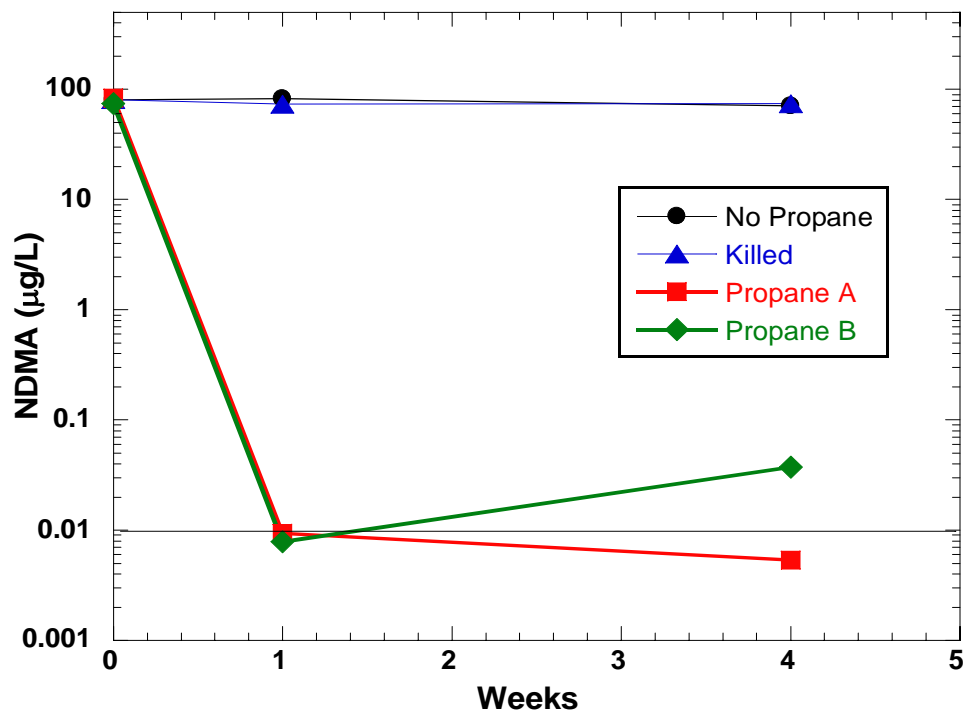
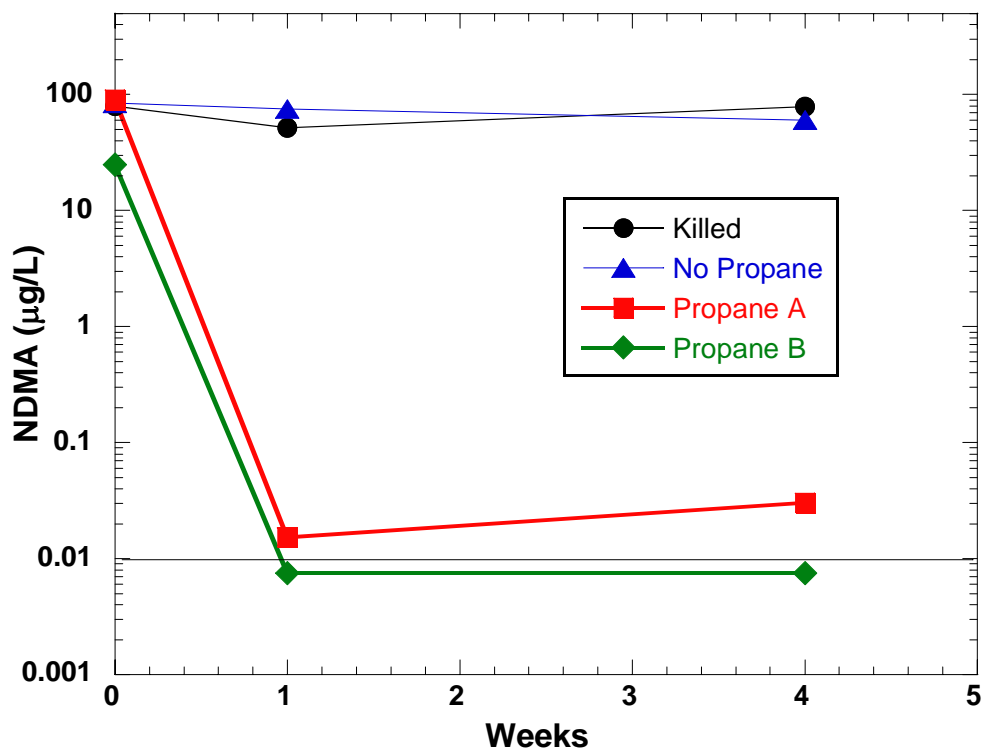


Figure 62. Biodegradation of NDMA in laboratory microcosms prepared with aquifer solids and groundwater from Air Force Site PJKS, CO. The microcosms (see Figure 7) were pre-incubated with oxygen and propane, and then spiked with NDMA to a starting concentration of $\sim 100 \mu\text{g/L}$.



4.6.3 Influence of TCE on In Situ NDMA Treatment using Propane Biostimulation

TCE is known to co-occur with NDMA at some military sites reflecting historical joint disposal of solvents and liquid rocket propellant in open-burn open-detonation areas, landfills, and at other locations. Because TCE is also a target for the broad-specificity oxygenase enzymes characteristic of both toluene-oxidizers and propanotrophs, the joint treatment of these co-contaminants by both classes of organisms has been examined. In pure culture, the degradation rate of NDMA by the toluene-oxidizer *P. mendocina* KR1 was appreciably reduced by TCE (see Figure 55). TCE degradation by the strain was, however, unaffected by NDMA. Similarly, the presence of TCE in a flow-through reactor system was observed to inhibit NDMA degradation by the propanotroph *R. ruber* ENV425 (see Section 4.4.3; Figure 53). Additional studies with this strain suggest that the inhibition is related to cell toxicity (e.g., by TCE-epoxide) (see Section 4.5.1). Besides pure culture and reactor-based studies, preliminary experiments were conducted during this quarter to assess the impact of TCE on NDMA metabolism by indigenous bacteria in aquifer samples (see next section). These studies are designed to provide insight on the potential impact of TCE on *in situ* NDMA degradation during propane biostimulation.

4.6.3.1 Materials and Methods: Influence of TCE on NDMA Mineralization

Aquifer microcosms were prepared using site materials obtained from the New Jersey and southern California sites. Twenty-five grams of homogenized aquifer solids and 25 mL of groundwater from each site were added to sterile 160 mL serum bottles. Inorganic nutrients (nitrogen, final concentration 60 mg/L, and phosphorus, final concentration 80 mg/L) were added to each bottle as a mixture of mono- and di-ammonium phosphate. Selected bottles for each site were enriched for propane oxidizing bacteria by adding 25% propane in the headspace for 28 days prior to inoculation with NDMA (and TCE in select treatments). After this incubation period, which was designed to stimulate indigenous propanotrophs, all propane was removed from the microcosms by flushing the headspace with air. Duplicate microcosms from the southern California site then received NDMA only (500 µg/L) or NDMA and TCE together (500 µg/L each). Duplicate microcosms that were not pre-incubated with propane also received NDMA and TCE together (500 µg/L each). A small amount of ¹⁴C-labeled NDMA and a base trap containing 1 mL of 0.5 N sodium hydroxide was added to each microcosm to quantify the ¹⁴CO₂ generated during mineralization of the ¹⁴C-NDMA. Duplicate microcosms prepared with aquifer materials from New Jersey received the same initial treatment with propane (28 days) and the same addition of NDMA and/or TCE as described for the southern California samples. In addition to these treatments, duplicate bottles received NDMA (500 µg/L), TCE (500 µg/L) and 20 % propane (in the headspace), and duplicate bottles that were not initially treated with propane received NDMA (500 µg/L). All bottles were incubated in the dark at room temperature on a rotary shaker operating at 50 rpm. Periodically, the

bottles were opened the NaOH in each trap was removed and replaced with fresh base. The quantity of $^{14}\text{CO}_2$ in each base trap was determined by scintillation counting.

4.6.3.2 Results and Discussion: Influence of TCE on NDMA Mineralization

Southern California

In the southern California microcosms, NDMA mineralization to $^{14}\text{CO}_2$ proceeded at a marginally slower rate when TCE was present as a co-contaminant at 500 $\mu\text{g/L}$ than when NDMA was the sole contaminant (Figure 63). During the initial 10 days of incubation average mineralization rate in the samples receiving NDMA only was 1.3 % per day and that in samples receiving TCE with NDMA was 1.0 % per day. These rates were each appreciably higher than the rate of NDMA mineralization in samples that were not preincubated with propane (0.5 % per day). After 20 days of incubation, 15% of the NDMA was mineralized in the microcosms that had received TCE, versus 19% in microcosms in which NDMA was the sole contaminant. In the control sample with TCE and NDMA that was not pre-incubated with propane, only 8% of the nitrosamine was degraded in 20 days. As expected based on previous results, the microcosms that had been enriched with propane prior to inoculation with NDMA exhibited more rapid degradation of NDMA when compared to the non-enriched controls. It should be noted also that none of the microcosms were amended with propane during the initial 20 days of incubation after addition of NDMA or NDMA with TCE.

At 21 days, an additional 1 mg/L of TCE was added to the microcosms that originally received TCE, and all microcosms except the control received propane in the headspace (1 % v/v). The addition of propane and/or TCE had no significant effect on the rate or extent of NDMA degradation in the treated samples (Figure 63).

New Jersey

Much like the southern California site, NDMA mineralization in the New Jersey samples occurred more slowly when TCE was present as a co-contaminant (Figure 64). The average mineralization rates (first 9 days) in the samples receiving NDMA only was 1.1 % per day and that in samples receiving TCE with NDMA was 0.72 % per day. The rate in the microcosms receiving TCE was not appreciably different than that in the control bottles (not pre-incubated with propane, rate was ~ 0.77 % per day with or without TCE). It should be noted also that none of the microcosms were amended with propane during the initial 20 days of incubation after addition of NDMA or NDMA with TCE. Propane was not added to these treatments in case the gas acts as an inhibitor of TCE biodegradation (it does not appear to inhibit NDMA degradation based on previous results) In the presence of propane, rates of NDMA mineralization in the presence of TCE were appreciably higher, averaging 1.2 % per day compared to 0.77 % per day without propane (Figure 64). Moreover, NDMA mineralization continued at a significantly higher rate in the propane-treated samples compared to the samples without

propane. Thus, propane served to stimulate NDMA mineralization even in the presence of TCE. These results differ from those in the propane-fed MBR in which TCE was inhibitory.

The results from both studies indicate that, while the presence of TCE is likely to slow the rate of NDMA degradation, the chlorinated organic is not likely to completely inhibit degradation at typical concentrations in groundwater (e.g., 500 µg/L). Moreover, if propane (the primary growth substrate) is present continuously during incubation, the impact of TCE on NDMA degradation may be reduced further. Based on the mineralization data with ^{14}C -NDMA, additional studies were conducted to quantify the losses of both TCE and NDMA in aquifer microcosms treated with propane to stimulate indigenous propanotrophs (See Section 4.6.3).

Figure 63. Influence of TCE on the mineralization of NDMA by indigenous bacteria in aquifer samples from a site in southern California. All microcosms except the controls were pre-incubated with propane for 4 weeks prior to addition of NDMA or NDMA/TCE to stimulate propane-oxidizing bacteria.

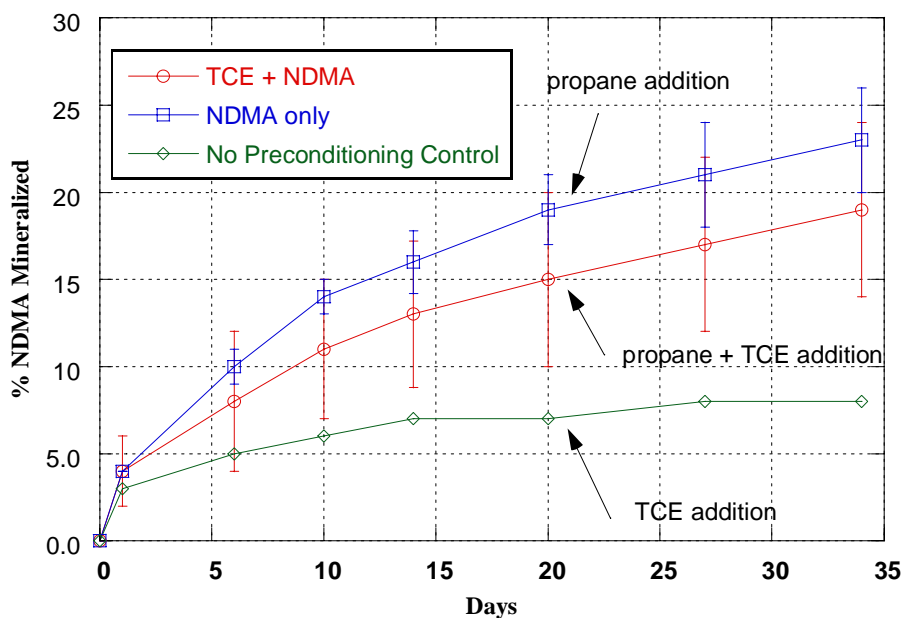


Figure 64. Influence of TCE on the mineralization of NDMA by indigenous bacteria in aquifer samples from New Jersey. All microcosms except the controls were pre-incubated with propane for 4 weeks prior to addition of NDMA or NDMA/TCE to stimulate propane-oxidizing bacteria.

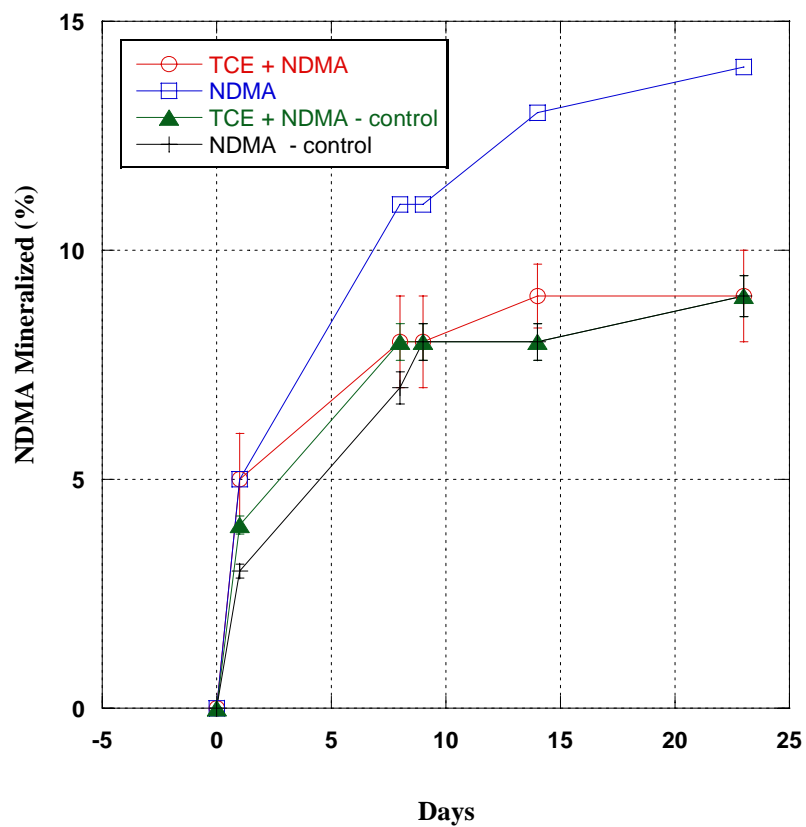
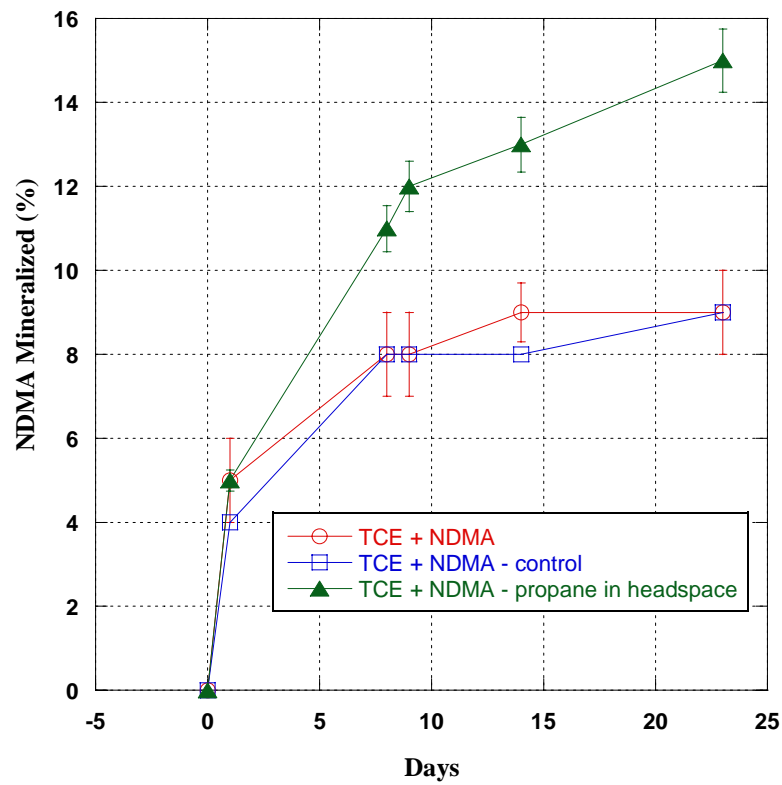


Figure 65. Influence of propane on the mineralization of NDMA in TCE-amended aquifer samples from New Jersey. All microcosms received TCE and NDMA. All except the control were pre-incubated with propane for 4 weeks prior to addition of the two contaminants. One set of samples continued to receive propane after contaminant addition, the others did not.



4.6.3.3 Materials and Methods: Influence of TCE on NDMA Treatment Levels: Large-Scale Microcosms

Results from earlier studies using radiolabeled NDMA (Section 4.6.3) indicate that, while the presence of TCE is likely to slow the rate of NDMA degradation, the chlorinated organic is not likely to completely inhibit degradation at typical concentrations in groundwater (e.g., 500 µg/L). Moreover, if propane (the primary growth substrate) is present continuously during incubation, the impact of TCE on NDMA degradation may be reduced further. Based on these results, studies were designed to quantify the losses of both TCE and NDMA at commonly found *in situ* concentrations in aquifer microcosms incubated with propane.

The treatment of NDMA using propane biostimulation was examined in microcosms prepared with site materials from Air Force Plant PJKS and the New Jersey site with favorable results (Figures 61-62). In each set of samples, NDMA was reduced from ~ 100 µg/L to < 50 ng/L within 1 week after pre-incubation with propane. No losses were observed in killed control samples or in those not receiving propane. At the conclusion of these experiments, additional groundwater was added to each microcosm to bring the final groundwater volume to two liters. Microcosms were then incubated with 20% propane and 80% oxygen gas in the headspace for two weeks. After this two-week conditioning period, the microcosm lids were replaced with airtight seals, and the microcosms were amended with NDMA and TCE at commonly found *in situ* levels (100 µg/L NDMA and 2 mg/L TCE). Treatments consisted of: (1) 100 µg/L NDMA plus 2 mg/L TCE in the liquid phase, 20% propane in the headspace; (2) 100 µg/L NDMA, no TCE, 20% propane in the headspace; (3) 100 µg/L NDMA plus 2 mg/L TCE in the liquid phase, no propane in the headspace; (4) 100 µg/L NDMA plus 2 mg/L TCE in the liquid phase, 2% formaldehyde, 20% propane in the headspace (killed control). Immediately following the addition of NDMA and TCE, the microcosms were shaken for 45 min to allow the NDMA and TCE to disperse evenly. Samples were then taken for TCE (20 mL VOA vial; EPA 8260) and NDMA analysis (500 mL aqueous sample filtered for preservation). Shaw's analytical laboratory performed the EPA 8260 analysis and samples for low-level NDMA analysis via GC/MS/MS were sent to Maxxam Analytics. Additional samples were collected after 1 week and 4 weeks of incubation. Once the sample was removed, the headspace was replaced with 20% propane and 80% oxygen and the microcosms were sealed tightly to prevent loss of TCE and propane.

4.6.3.4 Results and Discussion: Influence of TCE on NDMA Treatment Levels: Large-Scale Microcosms

NDMA was degraded from a starting concentration of 100 µg/L to less than 200 ng/L within one week in microcosms prepared from New Jersey site materials in the presence or absence of TCE (Figure 66). After 4 weeks of incubation, the NDMA concentration was 27 ng/L in the sample bottle receiving NDMA with TCE and 37 ng/L in the bottle receiving NDMA only (both samples with propane). Thus, there was no appreciable inhibition of TCE on NDMA biodegradation in these samples. No significant loss of NDMA was observed in the killed controls over the same 4-week period of time. There

was also a significant loss of NDMA in the live control bottle (no propane added) between 1 and 4 weeks of incubation. This may reflect aerobic co-oxidation of NDMA supported by a carbon source other than propane. Similar results were observed in samples from the New Jersey site early in this project. In this case, natural organic matter appeared to support biodegradative activity.

TCE was also reduced to below detection limits in the microcosm containing both NDMA and TCE within one week. There was a moderate decrease in TCE in the killed and no propane controls during this same time frame. The loss of TCE in these samples probably reflects volatile losses associated with flushing the headspace of the microcosms with oxygen. These data show that NDMA and TCE can be biodegraded simultaneously under simulated *in situ* conditions. Degradation of both contaminants was rapid, with NDMA concentrations reaching to < 40 ng/L within four weeks. These results are important for the eventual application of propane sparging for NDMA treatment in the field because TCE and other solvents frequently co-occur with NDMA.

Unlike samples from New Jersey, biodegradation of NDMA was not stimulated in the propane-fed microcosms prepared from Air Force Plant PJKS site materials during this experiment, irrespective of the presence or absence of TCE (Figure 67). In a previous study with these samples under identical conditions (Figure 62), NDMA was rapidly biodegraded from ~ 100 µg/L to < 30 ng/L within one week. However, the stimulation of NDMA degradation in the PJKS samples has been somewhat inconsistent throughout this project. This may reflect a limitation in a secondary factor (e.g., inorganic nutrients) or the composition of the microflora at the site (e.g., perhaps the numbers of propane oxidizers are very low). Additional site-specific studies are warranted to better understand the cause of the inconsistent results at this location.

Despite the inconsistent results from the Air Force Plant PJKS microcosms, the New Jersey microcosms demonstrated that NDMA degradation can occur in the presence of TCE at *in situ* levels of both contaminants. NDMA and TCE degradation was simultaneous and rapid, with NDMA levels reduced to ng/L levels within one week. These results indicate that *in situ* propane biostimulation may be a feasible remediation option for sites contaminated with both NDMA and TCE.

Figure 66. Biodegradation of NDMA (panel A) and TCE (panel B) by indigenous bacteria in aquifer samples from New Jersey. All microcosms except the controls were pre-incubated with propane for 2 weeks prior to addition of NDMA or NDMA/TCE to stimulate propane-oxidizing bacteria.

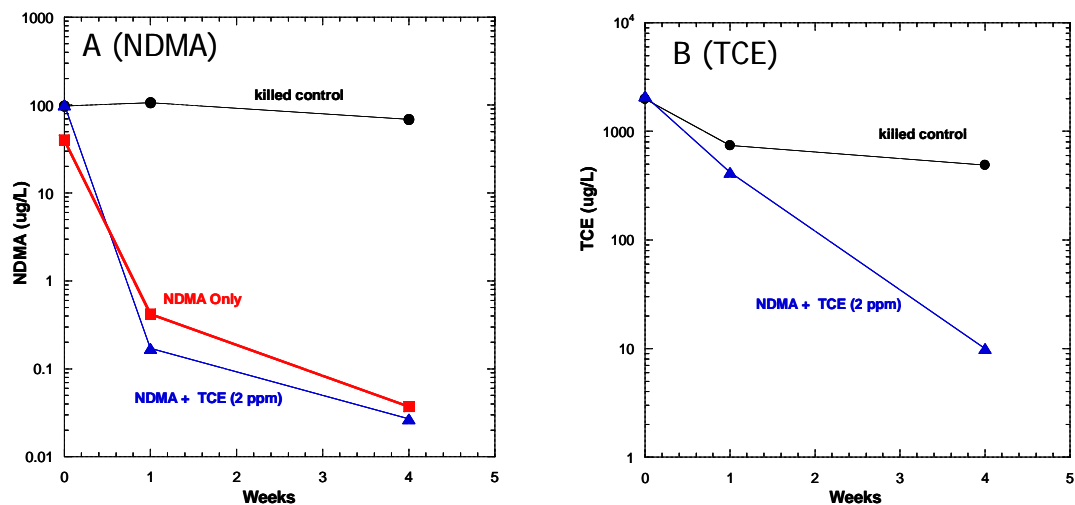
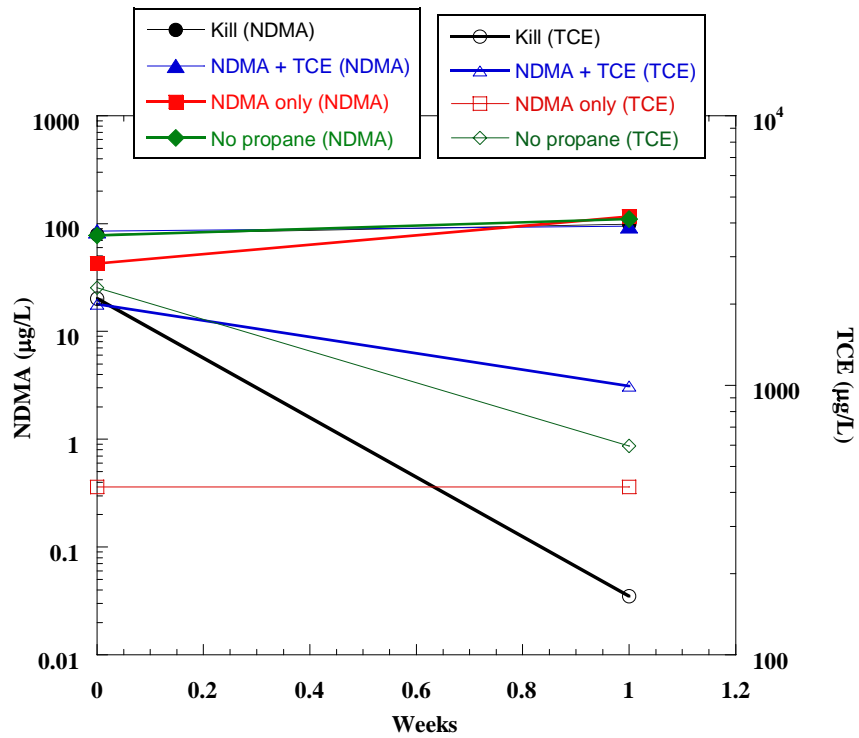


Figure 67. Biodegradation of NDMA and TCE by indigenous bacteria in aquifer samples from Air Force Plant PJKS, CO. All microcosms except the controls were pre-incubated with propane for 2 weeks prior to addition of NDMA or NDMA/TCE to stimulate propane-oxidizing bacteria.



4.6.4 Treatment of NDMA in Commercial Wastewater containing Dichloromethane (DCM) using ENV425

Four different NDMA-containing wastewater samples were obtained from a commercial manufacturing facility for use in this SERDP project. These samples contained NDMA at approximately 6-10 mg/L. The samples also contained DCM at concentrations ranging from 6 to 20 mg/L. Batch tests were conducted to assess whether strain ENV425 could biodegrade NDMA in any of these four samples.

4.6.4.1 Materials and Methods: NDMA Treatment in Commercial Wastewater

ENV425 was grown in basal salts medium (BSM-H) with propane in the headspace as a sole source of carbon and energy. Cells were washed and resuspended in the effluent samples to a final OD₆₀₀ of 0.8. Serum bottles (160 mL) were prepared with 100 mL of each effluent sample. One set of bottles were seeded with ENV425, one set remained without the culture (live control), and one set was inoculated with ENV425, then treated with formaldehyde (killed control). Propane (3% v/v) was added to the headspace of each bottle, and was replenished after each sampling event. The pH of each effluent sample was tested, and three were neutral (6.92 to 8.20). One sample with low pH (4.6) was amended with sodium bicarbonate until a pH of 6.8 was obtained. At the time of initial setup, and at selected time points thereafter, 3 mL of effluent was removed from each bottle to test for methylene chloride degradation via VOC analysis, and 2 mL was removed for NDMA analysis as described in previous reports.

4.6.4.2 Results and Discussion: NDMA Treatment in Commercial Wastewater

In Source water “I”, both NDMA and DCM were degraded to below detection within 6 days of incubation (Figure 68). In source water “O”, 46% of the DCM was degraded within 6 days, however, no significant NDMA degradation was observed in this same time frame (Figure 69). No significant degradation of either NDMA or DCM was noted in source waters “8” or “L” (Figures 70 and 71, respectively).

The data generated from this study suggest that ENV425 is capable of concurrent NDMA and DCM degradation. In groundwater from source “I”, NDMA degradation and DCM degradation occurred simultaneously. This suggests that, in this particular source water, the presence of DCM does not significantly inhibit NDMA degradation, and conversely, the presence of NDMA does not significantly inhibit DCM degradation. Thus, addition of the propanotroph ENV425 and supplemental propane may be a feasible alternative for remediation of groundwater contaminated with both the solvent dichloromethane and NDMA.

In source water “O”, 46% of the DCM was degraded within 6 days, however, no significant NDMA degradation occurred during the course of this study in this source water. No significant degradation of either of the compounds of interest in source waters “8” or “L”. Being that ENV425 is capable of degrading both contaminants

concomitantly, the lack of degradation observed in waters “O”, “8”, and “L” is most likely due to other contaminants within these waters that are either inhibitory to bacterial degradation or are lethal to ENV425. Degradation of DCM and NDMA in these waters may be feasible if other contaminants are identified and treated prior to bioaugmentation with ENV425.

Figure 68. Biodegradation of NDMA and DCM in “T” wastewater samples. Samples receiving culture ENV425 are denoted as “Bioaug” and uninoculated samples are denoted as “Control”.

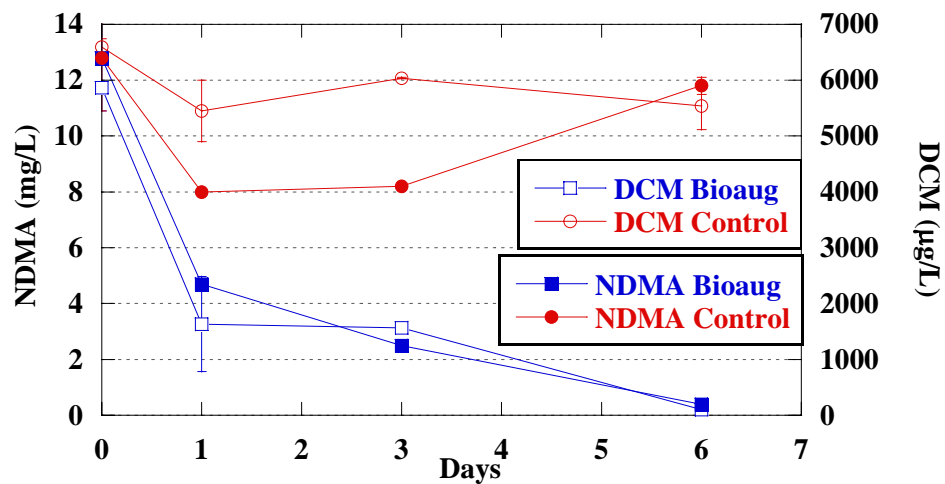


Figure 69. Biodegradation of NDMA and DCM in “O” wastewater samples. Samples receiving culture ENV425 are denoted as “Bioaug” and uninoculated samples are denoted as “Control”.

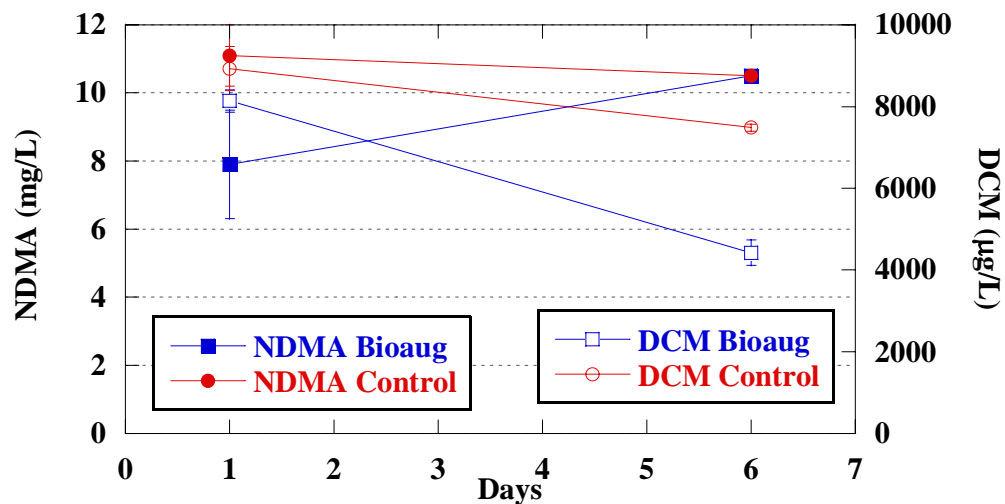


Figure 70. Biodegradation of NDMA and DCM in “8” wastewater samples. Samples receiving culture ENV425 are denoted as “Bioaug”, uninoculated samples are denoted as “Control”, and samples treated with ENV425 and formaldehyde are denoted “Killed”.

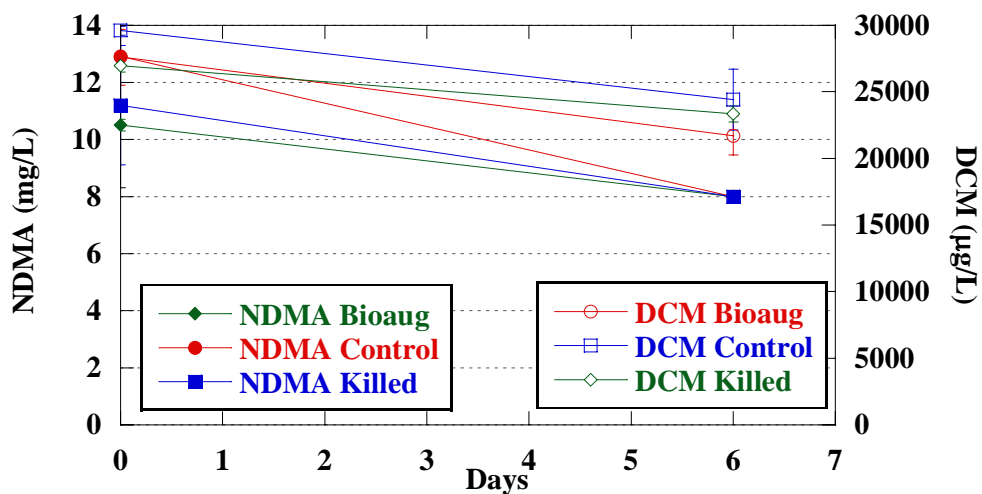
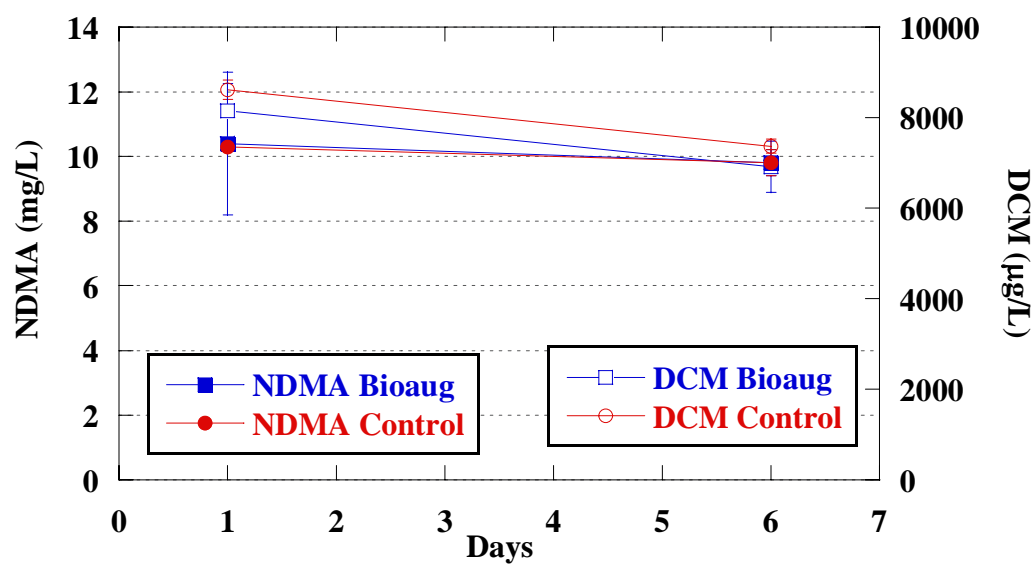


Figure 71. Biodegradation of NDMA and DCM in “L” wastewater samples. Samples receiving culture ENV425 are denoted as “Bioaug” and uninoculated samples are denoted as “Control”.



5.0 TECHNOLOGY TRANSFER

A manuscript from this work was published in *Applied and Environmental Microbiology* during October 2006, and three additional manuscripts are in preparation. A chapter in a textbook was also published as part of a training course for the US Air Force. Eleven presentations were given (or are planned) at national and international scientific meetings between 2005 and 2009.

5.1 Publications

Fournier, D., J. Hawari, S. H. Streger, K. McClay, and P. B. Hatzinger. 2006. Biotransformation of N-nitrosodimethylamine (NDMA) by *Pseudomonas mendocina* KR1. *Applied and Environmental Microbiology*, 72: 6693-6698.

Steffan, R.J., P. B. Hatzinger, and M.E. Fuller. 2007. Treatment Options for Emerging Contaminants. Pg. 229 – 238. In Hayes, S.W., C. T. Carper, and R. Williams (Eds), US Air Force Environment, Safety, and Occupational Health Training Symposium Textbook. US Air Force, Headquarters Air Combat Command, Langley AFB, VA, USA (available online: www.esvmpo.com).

Fournier, D., J. Hawari, S. H. Streger, K. McClay, and P. B. Hatzinger. 2009. Biodegradation of N-nitrosodimethylamine (NDMA) by the propanotroph *Rhodococcus ruber* ENV425. *Applied and Environmental Microbiology*, (In preparation).

Hatzinger, P.B., C. Condee, and A. P. Togni. 2009. Bioremediation of N-nitrosodimethylamine to part-per-trillion concentrations using a propane-fed membrane bioreactor. *Groundwater Monitoring and Remediation* (In preparation).

Hatzinger, P.B., and S. Streger. 2009. In Situ Bioremediation of N-nitrosodimethylamine using propane biostimulation. *Environmental Science & Technology* (In preparation)

5.2 Presentations

Streger, S., D. Fournier, J. Hawari, K. McClay, and P.B. Hatzinger. 2006. Biodegradation of N-Nitrosodimethylamine (NDMA) by Pure Cultures and in Environmental Samples. American Society for Microbiology, 106th General Meeting, Orlando, Fl, May 21 – 25.

Hatzinger, P.B., S. Streger, D. Fournier, and J. Hawari. 2006. Biodegradation and Bioremediation of N-Nitrosodimethylamine (NDMA) in Groundwater. The 22nd Annual

International Conference on Soils, Sediments, and Water. University of Massachusetts, Amherst, MA. October 16 – 19 (*platform presentation*).

Fournier, D., J. Hawari, S. Streger, K. McClay, and P.B. Hatzinger. 2006. Transformation of N-Nitrosodimethylamine (NDMA) by *Pseudomonas mendocina* KR1. Society of Environmental Toxicology and Chemistry, 27th North America Annual Meeting, Montreal, Canada, November 6 - 9.

Hatzinger, P.B., J. Hawari, D. Fournier, C. Condee, and S. Streger. 2006. Evaluating *In Situ* and *Ex Situ* Bioremediation Options for N-Nitrosodimethylamine in Groundwater. SERDP/ESTCP Partners in Environmental Technology, Technical Symposium and Workshop. Washington, DC, November 28 – 30, 2006 (*platform presentation*).

Hatzinger, P.B., J. Hawari, D. Fournier, and S. Streger. 2007. Biodegradation and Bioremediation of N-nitrosodimethylamine (NDMA). The Ninth International *In Situ* and On-Site Bioremediation Symposium, Baltimore, MD, May 7-10. (*platform presentation*).

Hatzinger, P.B. 2008. Bioremediation Options for N-nitrosodimethylamine (NDMA). AFCEE Technology Transfer Symposium, San Antonio TX, March 25-28. (*platform presentation*).

Hatzinger, P.B., J. Hawari, and D. Fournier. 2008. Evaluation of Bioremediation Options for NDMA Using Propane-Oxidizing Bacteria. The International Conference on the Remediation of Chlorinated and Recalcitrant Compounds. Monterey, CA, May 19-22 (*platform presentation*).

Hatzinger, P.B., C. Condee, S. Streger, and J. Hawari. 2008. Evaluating Biological Remediation Options for N-Nitrosodimethylamine (NDMA). SERDP/ESTCP Partners in Environmental Technology, Technical Symposium and Workshop. Washington, DC, December, 2-5.

Streger, S., C. Condee, K. McClay, and P.B. Hatzinger. 2008. Application of Propanotrophs for Treatment of N-Nitrosodimethylamine (NDMA) in Groundwater. American Society for Microbiology, 108th General Meeting, Boston, MA, May 21 – 25.

Hatzinger, P.B., C. Condee, S. Streger, and J. Hawari. 2009. Biodegradation and Bioremediation of N-nitrosodimethylamine (NDMA) in Groundwater. The Tenth

International *In Situ* and On-Site Bioremediation Symposium, Baltimore, MD, May 5-8, 2009 (*invited platform presentation*).

Hatzinger, P.B., S Streger, C. Condee, K. McClay, and J. Hawari. 2009. Biodegradation of N-nitrosodimethylamine. REMTEC, Remediation Technology Summit, Atlanta, GA, March 3-5, 2009 (*invited platform presentation*).

5.3 Training & Short Courses

Hatzinger, P.B., and A. Leeson. 2007. US Environmental Protection Agency (USEPA) National Association of Remedial Project Managers (NARPM) Training Course. 2007. DoD's Perspective on the Development of Innovative Approaches for the Treatment of Emerging Contaminants". 17th Annual Training Course, Baltimore, MD, May 21-25.

Steffan, R. J., P. B. Hatzinger, and M.E. Fuller. 2007. Treatment Options for Emerging Contaminants. US Air Force Environment, Safety, and Occupational Health Training Symposium. March 19-23, Pittsburgh, PA.

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